University of Alberta

Inhibition of *Listeria monocytogenes* on a Processed Meat Product with a Lactic Acid Bacterium

by

Denise R. Rivard

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

in

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DEDICATION

To my Grandparents,

W. Dave Connolly & Louise Evelin Connolly (Freed)

ABSTRACT

A psychrotrophic lactic acid bacterium (LAB) isolated from chilled, stored, vacuum packaged ground beef was identified as *Lactococcus lactis* (Mishra, M.Sc. thesis, University of Alberta, 1992) and claimed to have a broad inhibitory spectrum against spoilage and pathogenic bacteria. In the current study the LAB was inoculated onto the surface of wieners that had been contaminated with a cocktail containing three compatible strains of *Listeria monocytogenes.* The inoculated processed meat product was vacuum packaged and stored at 4°C for 14 weeks. Samples were taken at weekly intervals for the first 8 weeks and thereafter at bi-weekly intervals. A significant reduction in the number of *L. monocytogenes* on the wieners was observed in the presence of the LAB in both replicates of the study. Based on ribotyping and 16S ribosomal DNA sequencing, it was shown that the LAB was a strain of *Lactobacillus sakei* and not *L. lactis* as previously claimed.

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LIST OF ABBREVIATIONS

API – Analytical Profile Index

APT – All Purpose Tween

ATCC – American Type Culture Collection

AU – Activity unit

bp – Base pair

CHR – Chromosomal DNA

DNA – Deoxyribonucleic acid

DUP – University of Guelph Ribotyping Database Reference Collection

EDTA – Ethylenediaminetetraacetic acid

FDA – Food and Drug Administration

GRAS – Generally Recognized As Safe

h - Hour

HPB – Health Protection Branch, Canada

IU – International Unit

kb - Kilobase

LAB – Lactic Acid Bacteria

Lm – *Listeria monocytogenes*

MA – Modified atmosphere

MDa – Mega-Dalton

mg - Milligram

min - Minute

mL – Milliliter

MRS – de Man, Rogosa and Sharpe

OD – Optical density

PCR – Polymerase chain reaction

RAPD – Random amplification of polymorphic DNA

rDNA – Ribosomal DNA

RNA – Ribonucleic acid

s - Seconds

SDS – Sodium dodecyl sulphate

STE – TE buffer and sodium chloride

TAE – Tris-acetate. EDTA buffer

TE – Tris-HCl, EDTA buffer

TFA – Trifluoroacetic acid

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

UAL – University of Alberta Lactic Acid Bacteria collection

U.S.A. – United States of America

U.S.D.A. – United States Department of Agriculture

1. INTRODUCTION

Meat and meat products are highly susceptible to the growth of spoilage and pathogenic bacteria. Starting from the carcass directly after slaughter, each step in handling, chilling, drying, processing, and storage of the meat will determine which of the initial contaminating groups of bacteria will survive and dominate the microbial population (Holzapfel, 1998). Processing of meats to produce a range of products usually involves a heat treatment that dramatically alters the microbial flora of meat. In processed meats the surviving microflora and post-heating contaminants can markedly affect spoilage and safety. Refrigerated storage and the method of packaging also have a dramatic effect on the meat microflora. In refrigerated raw and processed meats under aerobic conditions the dominant spoilage microflora consists of putrefactive *Pseudomonas*-like bacteria; whereas, under anaerobic conditions (vacuum or anaerobic modified atmosphere storage with elevated carbon dioxide) a fermentative microflora dominates. This microflora consists primarily of lactic acid bacteria (LAB). Because of the low carbohydrate content of meat, LAB do not cause excessive acid production and hence souring of the meat. Under refrigerated anaerobic conditions this results in a dramatic extension of the storage life of meat. The association between meat products, microbes and storage conditions has resulted in the development of strategies to enhance the microbial quality of meat and to improve the safety of meat and meat products.

LAB is a heterogeneous group of Gram-positive bacteria that are readily isolated from anaerobically packaged meat and meat products. LAB are generally associated with a beneficial role in meats but they have also been associated with undesirable organoleptic changes. In recent years, their beneficial role has been extensively investigated. This has led to increased interest in using LAB as protective cultures in meat systems. Increasing knowledge of LAB and their metabolites in different food systems has resulted in the application of these protective cultures as biological food preservatives (biopreservatives).

Bacteria in any environment compete for nutrients and certain strains selectively dominate and exclude the presence of other strains in a mixed microbial population. Competitive exclusion techniques such as the Nurmi concept (Nurmi and Rantala, 1973) in live animals depends on the addition of a range of intestinal microorganisms to young animals, whereas in a meat system the equivalent concept depends on the addition of a characterized strain of LAB. To qualify for use as a biopreservative the added LAB must competitively outgrow the background microflora, it must not spoil the meat faster than the indigenous microflora, and it should inhibit pathogens of concern.

LAB have a variety of ways in which they interfere with microbial populations and allow them to dominate in a variety of meat products (Jay, 1996). This is important in vacuum packaged meat products where the environment selectively favours the growth of LAB that creates an unfavourable environment for Gram-negative spoilage organisms. The production and excretion of substances that are lethal or inhibitory to other organisms are well described in the literature. The antimicrobial agents such as organic (lactic and acetic) acids, hydrogen peroxide, carbon dioxide, diacetyl, and bacteriocins will be further discussed. Considerable emphasis in biopreservation has been placed on bacteriocinogenic LAB. Bacteriocins generally have a narrow antibacterial spectrum, however some bacteriocins such as nisin and pediocin have a relatively broad antibacterial spectrum against Gram-positive bacteria. Bacteriocins of Gram-positive bacteria are generally not considered to be active against Gram-negative bacteria. Therefore natural bacteriocinogenic LAB can be used to specifically target Gram-positive pathogens in vacuum packaged meat products.

The primary objective of this study was to evaluate the effectiveness of a lactic acid bacterium (strain UAL 185) obtained from Mishra (1992) to inhibit the growth and/or survival of *Listeria monocytogenes* in a processed meat system. *L. monocytogenes* is a common contaminant of raw and processed meats (Johnson, et al., 1990). It is a strain that causes the severe foodborne illness, listeriosis, and it is of considerable

concern to the food industry and in public health. Using *L. monocytogenes* as the targeted pathogen in the current investigation we evaluated the effectiveness of UAL 185 as biopreservative to enhance the safety of a processed meat product.

The strain of interest, UAL 185, was isolated from vacuum packaged, chilled, raw ground beef and was claimed to be a psychrotrophic strain of *Lactococcus* spp. by Mishra (1992). In chill stored meats it is important to select a LAB strain that can grow at low temperatures and effectively improve upon both the safety and quality of meat products. The reported broad activity spectrum of the strain and its ability to grow at low temperatures made this a promising organism for use as a biopreservative culture. To be effective as a biopreservative in a refrigerated meat product, the LAB strain must inhibit the growth of foodborne, psychrotrophic pathogenic bacteria, such as *L. monocytogenes* throughout the extended storage of the product. The strain UAL 185 was not fully characterized in the previous study (Mishra, 1992). If the strain proved to be successful for biopreservation of a processed meat product, the identity of the organism should be confirmed and the inhibitory substance(s) it produces should be characterized. In this study the organism was tested in a challenge study against three strains of *L. monocytogenes* inoculated onto wieners that were vacuum packaged and stored at 4°C for 14 weeks.

2. LITERATURE REVIEW

2.1 Lactic Acid Bacteria in Foods

Lactic acid bacteria (LAB) are described as a diverse group of Gram-positive, nonmotile, nonsporeforming, rod- and coccus- shaped organisms that ferment carbohydrates and higher alcohols to form chiefly lactic acid (Orla-Jensen, 1919). The LAB of importance in foods belong to the following genera: *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Weissella* (Stiles and Holzapfel, 1997). They are found in a wide variety of fermented beverages and foods, the intestinal tract of animals, plant materials, etc. (Sneath et al., 1986). LAB are reported to have practical significance in fermentations, bioprocessing, agriculture, food and more recently, medicine (Klaenhammer et al., 2002). The wide variety of applications for LAB has resulted in considerable efforts in research and product development. Aside from food applications there has been recent use of LAB in the production of industrial chemicals and biological products including biopolymers (*Leuconostoc* spp.), bulk enzymes (*Lactobacillus brevis*), ethanol, and lactic acid (*L. casei, lactis, delbrueckii, brevis*) (Gold et al., 1996; Hofvendahl and Hahn-Hagerdal, 2000). Another very interesting medical application is LAB as strong candidates for development as oral delivery vehicles for digestive enzymes and vaccine antigens (Wells et al, 1996; Pouwels et al., 1998; Steidler et al., 1998).

LAB are important as starter cultures in the manufacture of dairy, meat, vegetable, and bakery products and in many traditional food fermentations. Adventitious LAB from foods and those added to food products are generally considered to be safe and may possibility confer health benefits to consumers. In the United States, regulated LAB used in foods are assigned GRAS (Generally Recognized as Safe) status. LAB are a unique group of microorganisms because of their central role in food fermentations and preservation because they greatly affect the characteristic sensory attributes of the products in ways that are unattainable by other food processing methods. LAB have been traditionally used to improve aroma and texture and to prevent rapid spoilage of dairy and

meat products as well as vegetables and silage (Frazier et al., 1978; Beck, 1978). This review will consider the use of LAB in such foods, their application in food biopreservation; specifically in relation to meat products, the use of LAB to inhibit the growth of the foodborne pathogen *Listeria monocytogenes* in meats, and the unique antimicrobial compounds they produce during growth.

2.1.1 Lactic Acid Bacteria in Meat Products

The intrinsic properties of meat facilitate the growth and survival of many different microorganisms. High water activity, relatively neutral pH, fats, vitamins, minerals and proteins of meat cause it to be sensitive to microbial spoilage and to be a concern for safety because of the growth and survival of pathogenic microorganisms. Meat can well be described as many different microenvironments because there are different environments at the surface, near the surface and in the interior of meat. It is best to evaluate the quality and safety of meats by factoring in the different environments and the resulting microbes that each may support for growth and survival. The external factors applied to meat must also be considered, such as temperature, gaseous environment, and added chemical agents such as salt that dramatically affect the resulting microflora of a product. In meats, LAB constitute a part of the initial microflora that readily develops after meat is processed to fermented sausages, or chill stored and packed under vacuum or modified atmosphere (Hugas, 1998). LAB that grow at refrigeration temperatures are the natural strains that dominate meats (Stiles, 1996). Strains of LAB that are naturally present in meats and meat products are: *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc gelidum*, *Leuconostoc carnosum*, *Carnobacterium piscicola* and *Carnobacterium divergens*. The *Carnobacteria* spp. were initially reported in the literature as atypical lactobacilli (Holzapfel and Gerber, 1983).

The development of LAB as the predominant microflora in meats is often a desirable characteristic and is necessary to provide the unique properties of fermented meat products. The sensory changes in the products, along with the preservative effect due to the growth and proliferation of the LAB result in an increased shelf life of these meat

products. For example, during cold storage of red meat under modified atmosphere of increased carbon dioxide there is a change in microbial ecology due to a shift from predominantly putrefactive Gram-negative bacteria to the presence of primarily fermentative LAB. This is a desirable change because these putrefactive Gram-negative, *Pseudomonas*-like organisms in meat stored under aerobic conditions cause spoilage due to the production of off-odors, discoloration and slime. The change to a primarily LAB microflora produces a dramatic effect on the shelf life of meats (Dainty and Mackey, 1992). Vacuum-packaged beef stored at 0°C, or beef packaged in 100% CO_2 stored at 2°C, can have an extended storage life of 8 to 12 weeks (Egan, 1983; Nattress and Jeremiah, 2000). Spoilage characteristics such as off-flavours that make the product unacceptable are only observed after these extended storage times. The low carbohydrate content of meat and its strong buffering capacity make metabolic changes due to the growth of LAB virtually unnoticeable by sensory evaluation until some time after maximum population has been achieved (McMullen and Stiles, 1993). One study found that the predominating LAB on vacuum-packaged beef stored at 2°C were lactobacilli, carnobacteria, leuconostoc, and *L. raffinolactis* (Schillinger and Lücke, 1987). The study also reported that *L. sakei* and *L. raffinolactis* strains were the most competitive of the LAB microflora. The growth and presence of LAB have a definite affect on product quality and these can be desirable and necessary changes or undesirable spoilage characteristics. The metabolic products of LAB and the bacteria itself have a role in the preservation of foods, although it is important to realize that the uncontrolled growth of certain LAB can cause spoilage in meats and meat products.

2.1.2 Lactic Acid Bacteria in Dairy Products and Vegetables

The association of LAB and dairy products is well documented. The use of starter cultures was introduced early this century in the dairy industry, mainly to guarantee a uniform product quality in butter, cheese, and cultured milk products (Lindgren and Dobrogosz, 1990). LAB are important in ensuring and predicting product quality but are also used for their antagonistic effects in controlling spoilage and pathogenic bacteria in raw milk, fermented milk and cheese products. The effect of LAB antagonism in the control of spoilage bacteria and pathogens in raw milk, fermented milk and cheese

products has been summarized in the literature (Gibbs, 1987; Speck, 1981; Hurst, 1972). The inhibitory activity of LAB in dairy products has been attributed mainly to acids, bacteriocins, and related products produced by commonly used starter cultures (Lindgren and Dobrogosz, 1990)

LAB associated with milk fermentations preserve food by the low pH and lactic acid they produce, as well as bacteriocins, in particular nisin that has found widespread application as a food preservative (Delves-Broughton, 1990). Lactococci, lactobacilli, and leuconostocs have important associations with fermented milk products. In hard cheeses, lactococci are used as the inoculum, but adventitious lactobacilli such as *L. casei* play an important role in the ripening process (Stiles, 1996).

Lactobacilli are traditionally viewed as important microbes in the intestinal environment and therefore they have been added to milk products for their probiotic effect in the human intestine. Strains such as *L. acidophilus*, *L. casei* and *L. reuteri* have been added to milk products and health claims have been reported to be attributed to their presence in the products.

Despite their importance in the marketplace, vegetable products have not been subjected to the type of microbiological scrutiny applied to foods derived from milk and meat. There is increasing concern about the microbial safety of many vegetable products because of the potential for contamination with pathogenic microorganisms and subsequent lack of processing. The major safety concern with non-sterile, ready-to-eat products is the possible outgrowth of psychrotrophic pathogens, such as *L. monocytogenes*, in the absence of sensory defects (Brackett, 1992).

Vegetables provide an environment that is well suited to microbial growth, including pathogenic bacteria; however, other bacteria such as LAB can be desirable in fermented products or indicative of a spoiled product that would be considered unacceptable for consumption. Therefore, there is considerable interest and research surrounding the use of LAB in vegetable products to facilitate desirable sensory characteristics (vegetable

fermentations), their significant role in spoilage, as well as their use in inhibiting the growth of undesirable organisms in vegetable products. Bonestroo et al., (1992) described an example of using LAB to control spoilage and pathogenic bacteria in minimally processed foods. Grated carrots were inoculated with selected strains of *Lactobacillus plantarum* or *Pediococcus* spp. and incubated until the pH reached 3.8 (42°C for 7 h). The results showed that the combined effect of elevated incubation temperature and high lactic acid concentration inhibited the growth of the main spoilage organisms in grated carrots, the shelf-life was greatly enhanced and pathogenic microorganisms were inhibited (Bonestroo et al., 1992).

The possibility of preserving ready-to-use vegetables with bacteriocin-producing LAB has been investigated (Vescovo et al., 1995). Harris *et al.* (1992) proposed a paired culture consisting of a nisin-producing *L. lactis* and nisin-resistant *L. mesenteroides* for fermenting sauerkraut. Nisin suppressed growth of the spoilage organism *L. plantarum* in laboratory scale fermentations. The importance of bacteriocin production in fermented vegetables has also been addressed in the literature (Fleming et al., 1975; Harris et al., 1989).

2.2 Safety Concerns and Lactic Acid Bacteria

Adventitious LAB from the natural microflora of foods and those added to food products are generally considered to be safe and may possibility confer health benefits to consumers. In the United States, regulated LAB used in foods are assigned GRAS (Generally Recognized as Safe) status. Aguirre & Collins (1993), and Gasser (1994) reviewed the involvement of LAB in clinical human infections. Cases in which LAB were isolated from patients, were generally associated with an underlying disease condition that may have increased the potential for infection. The overall risk of infection by LAB is considered to be very low, particularly in view of their ubiquity in the food environment (Adams & Marteau, 1995).

Health benefits of LAB have been reported from the application of some strains as probiotic cultures. Beneficial properties of LAB attributed to the consumption of fermented dairy products have been reported such as anti-cholesterol activity, chemical reactions associated with reduction of nitrite (decreased formation of biogenic amines), improvements in immunological status, and decreased incidence of gastrointestinal disorders (Fernandes et al., 1987; Sissons, 1989). Reports have shown that certain cultures are advantageous to animal and human health in their ability to stabilize or normalize the microflora of the gastrointestinal tract (Fernandes et al., 1992) and they have been associated with anticarcinogenic activity and tumour control (Adachi et al., 1992).

2.3 Lactic Acid Bacteria and Biopreservation

Extensive research and interest surrounds the application and efficacy of LAB in foods as a form of biopreservation. Biopreservation refers to the extended storage life and enhanced safety of foods using the natural microflora and (or) their antibacterial products (Stiles, 1996). Biopreservation of foods by LAB depends on both the characteristics of the organisms and the nature of the food product. It is important to select strains of LAB that will produce the desired characteristics in the food product, such as reduced pH, sensory changes, or bacteriocin production to inhibit the growth of undesirable microbes.

Consumers demand minimally processed foods, prepared without or with very little added chemical preservatives and with a lower acid, sugar and fat content (Gould, 1992). In the production of these new, more "natural" products there is an added technical concern for health risks and spoilage problems because a food that lacks these components allows for rapid proliferation of a variety of pathogenic and spoilage microorganisms. Recent estimates from the Centers for Disease Control and Prevention in the United States suggest that there are 76 million cases of food-borne illness in the US each year that are estimated to cause as many as 5000 deaths (Mead et al., 1999). This is consistent with the increasing number of reported foodborne illnesses and intoxications and the recent significant outbreaks of foodborne diseases (salmonellosis, listeriosis and

haemorrhagic colitis) in the last 12 to 13 years (Jay, 1996). In the United States the cost of foodborne illness associated with *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus*, and *Toxoplasma gondii* is between $6.5 and $34.9 billion (Buzby and Roberts, 1997). To meet the needs and demands of the marketplace for safe and acceptable food, processors and scientists are investigating alternative methods of preserving foods that will extend their storage life, maintain quality, and enhance their microbial safety. Strategies for incorporating biopreservatives into foods include the use of LAB with proven antimicrobial activity as starter cultures or starter adjuncts, the use of biopreservative preparations in the form of a previously fermented product, and/or the use of semi purified, purified or chemically synthesized bacteriocins (Luchansky, 1999). Researchers continue to evaluate the efficacy of such biopreservative agents to enhance food quality and safety.

2.3.1 Using Biopreservation to target the *L. monocytogenes*

***Listeria monocytogenes*; the pathogen of concern**

L. monocytogenes is a gram-positive, psychrotrophic, and facultatively anaerobic organism that is widely distributed in the environment. *L. monocytogenes* has been recognized as an animal pathogen for over 70 years (Murray et al., 1926). *Listeria* spp. are widely distributed in soil (MacGowan et al., 1994), on vegetables (Beuchat, 1996), on meat (Jay, 1996), on milk (Kozak et al., 1996), and on fish (Ben Embarek, 1994) but it has only been in the last two decades that concern and research has been focused on the role of food in the transmission of human listeriosis. Listeriosis is case defined by the World Health Organization as the presence of *L. monocytogenes* in normally sterile body sites. Cases where systemic infections arise with *L. monocytogenes* are referred to as severe listeriosis. Listeriosis is a serious illness. This is reflected by a mortality rate of approximately 30% (Newton et al., 1992). This high case fatality rate coincides with the fact that severe *L. monocytogenes* infections account for the highest hospitalization rate of foodborne pathogenic bacteria. It most commonly takes the form of an infection of the uterus, the bloodstream or the central nervous system.

The primary groups at risk from listeriosis (in order of decreasing risk) are: organ transplant patients, patients with AIDS or HIV-infected individuals, pregnant women, patients with cancer, and the elderly (Rocourt, 1996). Severe *L. monocytogenes* infection during pregnancy can result in spontaneous abortion, stillbirth or birth of a severely ill baby due to infection of the fetus (McLauchlin, 1993). The three main routes of transmission of listeriosis are contact with wild or domestic animals, cross-infection of newborn babies in hospital, and foodborne infection. This review will focus on *L. monocytogenes* as a foodborne pathogen and the interest in using biopreservation to inhibit its growth and survival in meat products.

Foodborne outbreaks of *L. monocytogenes*; Concern for food safety

Since 1980 outbreaks of foodborne listeriosis have been associated with foods such as coleslaw, pasteurized milk, Vacherin cheese, Mexican-style soft cheese, Belgian pâté, smoked mussels, pork tongue in aspic, chocolate milk and raw-milk soft cheese. This wide variety of products is due to the growth-limiting parameters of the pathogen. *L. monocytogenes* is a psychrotroph, it's minimum and maximum growth temperatures are –0.4°C and 45°C, respectively (International Commission on Microbiological Specifications for Foods, 1996). The pH range in which growth occurs is 4.39 to 9.4 and *Listeria* spp. grow and survive in products with a water activity as low as 0.92. These growth parameters explain why many different foods have been associated with *L. monocytogenes* foodborne infections. Strains associated with foodborne outbreaks have differing characteristics, such as virulence factors and infectious dose, but the epidemic strains in a vast majority of foodborne outbreaks has been one of three serogroups; 1/2a, 1/2b or more frequently 4b. *L. monocytogenes* is a relatively common environmental organism and it is often found on raw or lightly processed foods. The hazard it causes must be assessed based on production processes that have inefficiently employed and consequently how this affected the safety of the products.

The first outbreak of foodborne listeriosis occured in North America (Nova Scotia, Canada) between 1981 and 1982 (Schlech et al., 1983). There were 41 cases of listeriosis

with 18 deaths. Epidemiological studies determined that the strain was of the serogroup 4b and it was linked to the consumption of coleslaw. The product was subjected to a variety of risk factors that could account for the outbreak. The cabbage was exposed to uncomposted sheep manure, that was the probable source of environmental contamination. Storage of the raw vegetables at refrigeration temperatures for an extended period of time allowed the *L. monocytogenes* to grow. The product was then consumed, unprocessed in a ready-to-eat formulation.

A more recent outbreak involved the consumption of ready-to-eat processed meat products. The incidence and growth of *L. monocytogenes* in processed meat products are well documented (Doyle, 1988; Glass and Doyle, 1989; Grau and Vanderlinde, 1992; Johnson et al., 1990). On October 12, 2002 there was a nationwide recall of approximately 27 million pounds of fresh and frozen ready-to-eat turkey and chicken products from Pilgrim's Pride/Wampler facility in southeast Pennsylvania (USDA., 2002). The recall states "WASHINGTON, Oct. 12, 2002- Pilgrim's Pride Corporation, doing business as Wampler Foods Inc., a Franconia, Pa., establishment, is voluntarily recalling approximately 27.4 million pounds of fresh and frozen ready-to-eat turkey and chicken products that may be contaminated with *Listeria monocytogenes*, the U.S. Department of Agriculture's Food Safety and Inspection Service announced today". The consumption of such products has been associated with at least 46 cases of listeriosis, 7 deaths and 3 miscarriages. A properly controlled cooking process was supposedly utilized which would achieve a significant reduction in the number of organisms, therefore it is assumed that there must have been a post-process contamination. This assumption is supported by reports that linked the listeria to floor drains and food contact surfaces in the facility. This highlights the importance of controlling and monitoring food processes to ensure that growth is controlled in both processing and in finished ready-to-eat product prior to consumption. The seriousness of *L. monocytogenes* infections and the demand for more "natural" and "minimally processed" food has resulted in the increasing interest in development of processes to ensure the safety and quality of foods. More specifically, numerous studies have investigated the use of

biopreservative techniques to inhibit the growth and survival of *L. monocytogenes* in meat products.

Targetting *L. monocytogenes*; Methods of biopreservation in meats

Food manufacturers continue to develop strategies to increase the safety of meat and meat products by employing technologies to reduce the number of microorganisms in meat. Since 1993, meat carcass sanitization has included stream pasteurization, the use of trisodium phosphate, and spraying with one or more organic acids (Jay, 1996). These treatments and the production of minimally processed products could create a hazard for health because pathogens can proliferate without a competitive microflora. This is mainly due to the fact that these strategies are not selective for the pathogenic microbes and therefore other bacteria will also be inhibited. The inactivation of the growth of the natural microflora of meat is a dangerous scenario because it creates a product with little competition for the growth of pathogenic microorganisms. Strategies must be developed that are directed at pathogens, such as *L. monocytogenes*.

Biopreservative agents produced by LAB have been investigated for use in foods, however to date, there are few available formulations used by food processors. Diacetyl and lactic and acetic acids are listed as GRAS and will be introduced later in the review. Nisaplin®, a commercial product containing nisin from *L. lactis*, and Microgard® that is produced by propionibacteria (that are not members of the LAB group of bacteria) are approved as food preservatives by the Food and Drug Administration (FDA). Microgard® is a commercially available product of skim milk fermented by *Propionibacterium freudenreichii* subsp. *shermanii* and then pasteurized. It is used in about 30% of cottage cheese in the United States (Daeschel, 1989). The antimicrobial activity is attributed to a heat-stable polypeptide that is protease-sensitive and the production of propionic acid (Al-Zoreky et al., 1991), and is active against Gram-negative bacteria, Gram-positive bacteria, yeasts, and molds. Microgard® decreased growth of inoculated strains of *L. monocytogenes* on steam-sterilized fresh crab meat for 6 days at 4°C by 0.5 to 1 log but after 6 days the numbers increased to the original level of inoculation (Degnan et al., 1994).

LAB have been extensively investigated for their use in the biopreservation of meat products. The natural preservatives produced by LAB such as organic acids, hydrogen peroxide, diacetyl, and bacteriocins will be discussed later in the review. The use of LAB and/or their antimicrobial products has already been demonstrated to extend the storage life and safety of chilled, MA (modified atmosphere) stored meats (McMullen and Stiles, 1996). It has also been shown that a strain of LAB can delay the spoilage of chill stored, vacuum packaged meat for up to 8 weeks (Leisner et al., 1996). The application of LAB in biopreservation expands beyond increasing the storage life of meat products to the inhibition of foodborne pathogens. Much research has focused on targeting the pathogen *L. monocytogenes* because of its presence in many products, the severity of infection and its ability to survive and grow at refrigeration temperatures.

2.4 Antimicrobial Agents Produced by Lactic Acid Bacteria

LAB produce a variety of antagonistic factors that include metabolic end products, antibiotic-like substances and antibacterial proteins, termed bacteriocins (Klaenhammer, 1988). This review will discuss the antimicrobial agents produced by LAB and their application in food systems to extend the storage life or to inhibit pathogenic microorganisms in foods. The primary preservative action of LAB is the reduction of pH by carbohydrate fermentation and the formation of acidic end products (Daeschel et al., 1989). The secondary preservative action is due to the production of inhibitory agents such as hydrogen peroxide, diacetyl, bacteriocins, non-proteinaceous antimicrobial agents, and secondary reaction products. The organic acids, hydrogen peroxide and diacetyl produced by LAB will be briefly discussed but the review will focus on the production and application of bacteriocins and novel antimicrobial compounds of a non-proteinaceous nature, such as reuterin.

2.4.1 Organic Acids

Fermentation of the carbohydrates, glucose, glycogen, glucose-6-phosphate and small amounts of ribose, produce organic acids, by glycolysis or by the hexose monophosphate

shunt pathway (Holzapfel, 1998). Carbohydrate metabolism serves as the main source of energy for most Gram-positive bacteria, and with the LAB it involves substrate-level phosphorylation. The most common classical preservative agents are the weak (organic) acids, such as lactic and acetic acids. Aside from a preservative role, it has been suggested that acetic acid and lactic acid may serve as possible parameters for estimating microbiological quality of packaged meat (Drosinos and Board, 1995).

Lactic acid is the primary end product of homofermentative and heterofermentative LAB. It is the typical end product of sausage, sauerkraut, olive, yogurt, and many dairy fermentations. It reduces the pH to a level where many microorganisms will be inhibited or destroyed. Putrefactive (clostridia and pseudomonads), pathogenic (salmonellae and *Listeria* spp.) and toxigenic (*Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum*) microbes are affected by the antagonistic action of organic acids (Holzapfel et al., 1995). The mechanism of inhibition is the ability of the undissociated acid to diffuse into the bacterial cell, thereby reducing the intracellular pH which results in the substantial and efficient decrease in metabolic activity of the cell. The undissociated form of the acid is fat soluble and can passively diffuse through the lipophilic cell membrane. Subsequently, at the higher pH inside the cell, the acids dissociate resulting in the release of charged anions and protons that cannot cross the plasma membrane (Brul & Coote, 1999). Inhibition of growth by weak acids is attributed to a number of actions including, membrane disruption (Bracey et al., 1998; Stratford and Anslow, 1998), inhibition of essential metabolic reactions (Krebs et al., 1983), stress on intracellular pH homeostasis (Salmond et al., 1984; Cole and Keenan, 1987; Bracey et al., 1998) and the accumulation of toxic anions (Eklund, 1985).

Acetic acid has a higher dissociation constant (pKa 4.75) than lactic acid (pKa 3.10) and therefore elicits a stronger inhibition at a given molar concentration. Lactic and acetic acids are produced from hexoses in equimolar amounts by heterofermentative LAB. Acetate is usually produced in smaller amounts as a metabolic end product but it is a vital factor in combination with lactic acid. This combined effect is apparent in the establishment of the initial LAB population during the fermentation of many products

such as sauerkraut and silage (Buckenhüskes et al., 1990). Under specific conditions of hexose limitation and/or oxygen availability, homofermentative LAB (e.g. pediococci, lactococci and most *Lactobacillus* spp.) may decompose lactic acid to acetic acid, formic acid and/or CO_2 (Kandler, 1983). Acetic acid is also widely used as a food additive for the preservation and flavouring of foods.

2.4.2 Hydrogen Peroxide

Hydrogen peroxide is produced during the growth of some LAB in the presence of molecular oxygen together with lactate, pyruvate and NADH by flavin enzymes (Kandler, 1983). It is formed by several different mechanisms but it accumulates because the organisms do not possess catalase to break it down. The formation and accumulation of hydrogen peroxide by lactobacilli with an antagonistic effect was first reported against *Staphylococcus aureus* (Dahiya & Speck, 1968) and *Pseudomonas* spp. (Price & Lee, 1970). These microorganisms are 2 to 10 times more sensitive to hydrogen peroxide than most LAB. This was initially demonstrated by Wheater *et al.* (1952) where the minimum inhibitory concentration (MIC) for hydrogen peroxide against *Lactobacillus lactis* was 125 μg/mL whereas the MIC for *S. aureus* was as little as 5 μg/mL. Hydrogen peroxide may activate the lactoperoxidase system that is indigenous to fresh milk with the formation of hypothiocyanite and other antimicrobial products active against both bacteria and fungi (Reiter & Härnulv, 1984). While the mechanism whereby hydrogen peroxide kills spores is not known, it kills vegetative cells of bacteria (Ananthaswamy & Eisenstark, 1977; Imlay & Linn, 1988) and fungi (Frankenburg et al., 1993) by damaging the DNA. The inhibitory effect is mediated through the strong oxidizing effect on membrane lipids and cell proteins (Morris, 1976). Regulatory authorities in the United States allow direct addition of hydrogen peroxide to food products such as raw milk for the preparation of certain cheeses, whey intended for use in modified whey preparation, corn starch and dried eggs, and for the decontamination of packaging materials (Toledo, 1975; Busta and Foegeding, 1983; Russell, 1991; Juven and Pierson, 1996). The amount of hydrogen peroxide tolerated in foods depends on the product type, and may be detrimental to the sensory quality of the product (Holzapfel et al., 1995), such as in meats where hydrogen peroxide causes greening of myoglobin. Strains, especially of *W.*

viridescens and *Leuconostoc* spp., but also of *L. fructivorans*, *Enterococcus faecium* and *Enterococcus faecalis*, have been associated with this type of spoilage (Grant et al., 1988).

2.4.3 Carbon Dioxide

The endogenous accumulation of carbon dioxide in fermented foods occurs as a result of natural respiration of cells as well as a metabolic product of microbial growth. Carbon dioxide has a dual role in the preservation of food products. First, it creates an anaerobic environment by replacing the molecular oxygen in the product, thereby inhibiting the growth of strict and some facultative aerobic bacteria. Secondly, carbon dioxide has its own antimicrobial activity (Clark and Takacs, 1980). The protective role of carbon dioxide is especially important in the fermentation of silage and vegetables to prevent growth of molds (Lindgren and Dobrogosz, 1990), and in the microbial environment of vacuum packaged meat products where it may be stimulatory to the growth of some bacteria. The production of CO_2 from the metabolism of lactate by propionibacteria in Swiss cheese production is a desirable characteristic of the finished product.

2.4.4 Diacetyl

Diacetyl (2,3 butanedione) is a metabolic product that is formed from pyruvate in the presence of citric acid (Kandler, 1983). Certain species and strains of all genera of LAB produce diacetyl. Diacetyl is the characteristic aroma of butter and it gives cultured dairy products their characteristic buttery odor. It is antimicrobial at 200 μg/mL for yeasts and gram-negative bacteria and at 300 μg/mL for most gram-positive bacteria (Jay, 1982). LAB are not inhibited at concentrations less than 350 μg/mL (Daeschel et al., 1989). In 1936 Jalander reported the inhibitory activity of diacetyl against a large number of microorganisms including *Mycobacterium tuberculosis* and in 1978 Spillmann confirmed the antibacterial activity of diacetyl against *Escherichia coli* with as little as a few parts per million of diacetyl (Lindgren and Dobrogosz, 1990). Bioactivity increased with the decreasing pH. Diacetyl has GRAS status with the FDA but its utility in food products is limited because large amounts are generally necessary for preservation, it is a highly

volatile aromatic compound, and it has an intense aroma that limits its use in most food products.

2.4.5 Bacteriocins

2.4.5.1 Definition and Classifications

In 1925 Gratia reported the first discovery of a highly specific antibiotic (principe V) produced by a strain of *E. coli* that was active against other strains of the same species (Daw and Falkiner, 1996). From this initial observation, the study of so-called colicins by many species of *Enterobacteriaceae* began and in time this interest expanded to bacteriocins produced by lactic acid bacteria. In 1928 Rogers reported an antibacterial substance produced by strains of *Lactococcus lactis* (van Belkum and Stiles, 2000). This substance, nisin A was characterized (Gross and Morell, 1971), and is the most studied and well-characterized bacteriocin of the LAB group.

Bacteriocins of LAB are ribosomally produced antimicrobial polypeptides or proteins that in their mature form, have an antibacterial effect against a narrow spectrum of closely related bacteria (Jack et al., 1995). Defined across Gram-positive and Gram-negative bacterial boundaries, bacteriocins represent a large class of heterogeneous bacterial antagonists that vary considerably in molecular weight, biochemical properties, range of sensitive hosts and mode of action (Klaenhammer, 1988). Many LAB produce bacteriocins, in fact, it was suggested by Jack et al. (1995) "all bacteria produce bacteriocins". For the purpose of this review, only bacteriocins produced by LAB will be discussed.

Based on biochemical and genetic characterization of many bacteriocins, Klaenhammer (1993) described four distinct classes of LAB bacteriocins:

Class I – Lantibiotics; small, membrane-active peptides containing unusual amino acids lanthionine, β-methyl-lanthionine, and dehydrated residues.

Class II - Small, heat-stable, nonlanthionine-containing membrane-active peptides; subgrouped as follows:

Class IIa: *Listeria*-active peptides with a consensus sequence (YGNGVXC) motif in the N-terminus

Class IIb: Complexes consisting of two proteinaceous peptides for activity

Class IIc: Thiol-activated peptides requiring reduced cysteine residues for activity

Class III - Large, heat-labile proteins

Class IV - Complex bacteriocins requiring protein and other chemical moieties for activity

There have been many other proposed classification systems for bacteriocins. In 1996, Nes et al. suggested that class II bacteriocins should be regrouped in that the class IIc be renamed "bacteriocins produced by the general secretory (*sec*) pathway". This was however further modified, as new knowledge and understanding arose surrounding recent research reports. A review by van Belkum and Stiles (2000), proposed that the class II bacteriocins be reclassified according to the number and position of disulfide bridges in the peptide. The subdivision of the class II bacteriocins was as follows:

Class IIa: Cystibiotics with two disulfide bridges

Class IIb: Cystibiotics with one disulfide bridge resulting from two cysteine residues in the N-section of the peptide

Class IIc: Cystibiotics with one disulfide bridge that spans the N- and C- sections of the peptide

Class IId: Peptides containing one (thiolbiotics) or no cysteine residues

Class IIe: Two-peptide bacteriocins

Class IIf: Atypical bacteriocins

The class I and II bacteriocins are of greatest interest in food applications due to their activity spectra and their potential ability to remain active in food products.

2.4.5.2 Application of Bacteriocins in Meat Products

The use of bacteriocins and bacteriocinogenic LAB in food products has been investigated for their application in microbial food safety. Investigations have also explored the applications of bacteriocins in hurdle technology, which utilizes synergies of combined treatments to more effectively preserve food. The study of bacteriocins as

preservatives in foods can be misleading and confusing unless they have been fully characterized (Stiles, 1996). The most studied bacteriocins in meat and meat products include nisin A, A,P and K, and leucocin A but especially pediocin PA-1/AcH (Hugas, 1998). It is important to realize that work in this area is of great importance because the application of bacteriocins in meat must be assayed in a meat system, and not only laboratory media because the environment of meat is very complex and the efficacy of bacteriocins in an applied meat system may be very different than what is observed *in vitro* situations.

Nisin has been in use as a food preservative for over 50 years and is approved as a food additive in over 46 countries for use in processed cheese, dairy products and canned foods (Delves-Broughton et al., 1996). The approval and applicability of nisin has been mainly observed in dairy products. In a cottage cheese spiked with 10^4 cfu/g *L. monocytogenes*, the addition of 2000 IU/g nisin resulted in a 1000-fold decrease in *L. monocytogenes* after 7 days storage at 20°C, compared to a 10-fold decrease in the control (Ferreira and Lund, 1996). Many food products have a natural microbial environment that contain nisin-producing strains of *Lactococcus lactis* and other bacteriocinogenic LAB. Therefore, consumers have been undoubtedly consuming bacteriocin producing organisms for centuries. A study of 40 wild-type strains of *L. lactis* showed that 35 produced nisin (Hurst, 1981). A limitation however, is that the producer organism does not grow in chilled meats. The ineffectiveness of nisin in meats is attributed to reactions with meat components, poor solubility, sensitivity to food enzymes, uneven distribution in the products, interaction with phospholipids, and lack of stability (Scott and Taylor, 1981; Bell and De Lacy, 1986; Henning et al., 1986; Stringer et al., 1995, Delves-Broughton et al., 1996). A study by Rose et al., (1999) showed that nisin is likely inactivated in raw meat by an enzymatic reaction with glutathione, a compound commonly found in animal and plant tissue.

It has been found that sprayed nisin (Ambicin®, 5000 AU/mL) has been effective for the decontamination of meat surfaces by a reduction of 2 to 3.5 log cfu/cm^2 on both lean and adipose tissue (Cutter and Siragusa, 1994). A combination of nitrite and 1000 to 10,000

IU/g of nisin was effective against *Clostridium* and some other Gram-positive pathogens such as *Listeria* and *Staphylococcus* in frankfurters, pork slurries and raw meat (Rayman et al., 1983; Chung et al., 1989). Investigations of nisin on meat products have concluded that the required dose necessary for effective inhibition is uneconomical and exceeds the acceptable daily intake levels. Studies will continue to improve the efficacy of nisin in meat systems because its activity spectrum is a desirable characteristic, however much work is needed and other characterized or novel bacteriocins may be more applicable in meats.

The literature reports many studies on the use of *Pediococcus* spp. and pediocin in raw, cooked and fermented meat products. Compared to nisin, pediocin is more suitable for use in meat products; however, pediococci are not indigenous to meats and therefore are unable to grow and produce bacteriocins in refrigerated products (Hugas, 1998). A study by Luchansky et al., (1992) fermented turkey summer sausage with *P. acidilactici* JBL1095, producing pediocin or the negative variant JBL1350. In the 12 hour fermentation equal amounts of acid were produced; however, a significant bacteriocin effect was observed in that *L. monocytogenes* inhibition was approximately 3.4 log with JBL1095 compared with 0.9 log with JBL1350. The sausages were inoculated on the surface with *L. monocytogenes* and *P. acidilactici* (Luchansky et al., 1992) and stored at 4°C and 25°C, it was shown that at 4°C the bioprotective cultures were not able to inhibit the pathogen but at 25°C *P. acidilactici* inhibited *Listeria*; showing a bioprotective effect in conditions of temperature-abuse. In other *in situ* assays with fresh meat and fermented sausages, pediocin has been able to reduce or at least stabilize the indicator strains (Nielsen et al., 1990; Foegeding et al., 1992). Attachment of *Listeria* spp. was less in meat treated with bacteriocin, and activity of the bacteriocin could be detected on the meat after 28 days of refrigerated storage (Nielsen et al., 1990).

The Wisconsin Process for cured bacon uses a pediocin-producing strain of *P. acidilactici* with added sucrose (Tanaka et al., 1980). It is approved by the U.S.D.A. for its application in reducing nitrite levels in cured bacon and has been shown to aid in the prevention of botulinum toxin production by outgrowth of *C. botulinum*. The process has

also been adapted for use in low-acid refrigerated food such as chicken salad at pH 5.1 (Hugas, 1998). The Wisconsin Process is an example of the application of the hurdle concept in food safety. The combined effect of different preservative actions may be more effective than any of the technologies used separately. The use of pediococci as protective biopreservative cultures has proven to be successful in fermented meat products but not in fresh meats stored at refrigeration temperatures.

The use of 2,400 AU of purified pediocin per gram resulted in 2.8 log cfu/g *L. monocytogenes* in raw chicken after 28 hours of storage at 5°C, whereas the uninoculated chicken sample had high levels of 8.1 log cfu/g (Goff et al., 1996). In this case it was concluded that purified pediocin would be effective in reducing *L. monocytogenes* levels in a raw chicken product, stored at refrigeration temperatures. The disadvantage of pediocin is that it is not yet approved as a food additive in the United States and therefore must be incorporated as an organism, not as a purified product in a food product. However, as mentioned, the producer organism is unable to grow and produce pediocin in meat products at low temperatures.

Indigenous organisms in meats, such as LAB, may be the most promising for use in the biopreservation of meat products. It is desirable to select for organisms isolated from meats and those that can grow at refrigeration temperatures and produce bacteriocins. Organisms such as *Lactobacillus sakei* Lb706, producing sakacin A (Schillinger and Lücke, 1989 and Schillinger et al., 1991). *Carnobacterium* piscicola LV17, producing carnobacteriocins (Worobo et al., 1994; Quadri et al., 1994) and *Leuconostoc gelidum* UAL 187 producing leucocin A (Hastings et al., 1991), will be examined for their applicability in meat products. It is known that these bacteriocins do not have a broad spectrum comparable to nisin or pediocin but grow at refrigeration temperatures and inhibit the targeted foodborne pathogen of concern, *L. monocytogenes*.

In a study by Schillinger and Lücke (1991), *L. sakei* Lb706 and a bacteriocin-negative variant, *L. sakei* Lb706-B, were added to samples of pasteurized minced meat that was inoculated with a sensitive strain of *L. monocytogenes*. The bacteriocin-producing strain

reduced the count and delayed the growth of *Listeria* more effectively than the bacteriocin negative strain. Sakacin K is a bacteriocin produced by *Lb. sakei* and is identical in bacteriocin composition to sakacin A and curvacin A, produced by *Lb. curvatus* (Axelsson and Holck, 1995; Tichaczek et al., 1993). Sakacin K-producing *Lb. sakei* CTC494 added to dry fermented sausages inhibited *Listeria* spp. in a model and meat system (Hugas et al., 1995). The authors recommended the use of the bacteriocinogenic strains of *Lb. sakei* for use as a bioprotective starter in fermented meat products.

Carnobacteria spp. isolated from meats have been shown to have antilisterial activity, although few studies have evaluated their effectiveness as a biopreservative in a meat product. *C. piscicola* LV17 produces bacteriocins, encoded on plasmid DNA, that are active against *L. monocytogenes* strains. However, reports have indicated that carnobacteriocins have not been detected in meat samples and may not be effective in fresh meat products due to unpredictable growth and lack of bacteriocin production (McMullen and Stiles, 1996). The activity spectrum of bacteriocins isolated from *Carnobacteria* spp. generally do not include all spoilage or pathogenic organisms of concern in meats (Stiles and Hastings, 1991). An exception to this is the broad activity spectrum of piscicolin 126 isolated from the strain *Carnobacterium piscicola* JG126 (Jack et al., 1996). Future work into the isolation, characterization and application of bacteriocins produced by *Carnobacteria* spp. should be interesting and rewarding fields of study.

Bacteriocin-producing *Leuconostoc* spp. have been isolated from various foods such as cheddar cheese, vacuum packaged vienna sausages, and vacuum packaged meat (Hastings et al., 1994). Their widespread presence in food products may indicate their effectiveness in biopreservation. It was reported that inoculation of a leucocin A-producing *L. gelidum* UAL 187 delays spoilage by a *L. sakei* strain for up to 8 weeks, in vacuum packed beef (Leisner et al., 1996). The organoleptic changes, characteristic of spoilage due to the presence of *Leuconostoc* spp. may limit their use in meat products. An example, is the limitation of the application of *L. gelidum* UAL 187 to processed

meats because of the production of polysaccharides from sucrose and its heterofermentative metabolism that results in the production of carbon dioxide (McMullen and Stiles, 1996).

2.4.6 Non-proteinaceous antimicrobial agents

The most extensively characterized non-proteinaceous antimicrobial substance produced by a lactic acid bacterium was termed reuterin and was discovered by Axelsson et al. (1989). Reuterin is a broad-spectrum antimicrobial substance produced by *Lactobacillus reuteri*, a prominent member of the heterofermentative *Lactobacillus* population in the gastrointestinal tract of humans, swine, poultry and other animals (Kandler et al., 1980; Kandler and Weiss, 1986). In 1962 Lerche and Reuter first isolated *L. reuteri* but classified at the time as *Lactobacillus fermentum* Type II (Lindgren and Dobrogosz, 1990).

The production of reuterin is dependent upon the availability of glycerol during growth of the producer organism. Resting cells of *L. reuteri* were found to convert glycerol (or glyceraldehyde, reduced to glycerol), in the presence of coenzyme B_{12}, into the potent, broad-spectrum antimicrobial substance (Axelsson et al., 1989). Chemically, reuterin is reported as a low molecular weight (less than 200 g/mole), water soluble, non-proteinaceous, neutral compound; β-hydroxypropionaldehyde. Nuclear magnetic resonance studies determined reuterin to be an equilibrium mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of β-hydroxypropionaldehyde (Talarico and Dobrogosz, 1989). This was later confirmed by chemical synthesis of the antimicrobial compound. The most interesting characteristic of reuterin is its broad spectrum of antimicrobial activity. It is active against many Gram-positive and Gram-negative bacteria, yeast, fungi, and protozoa. Organisms of public health significance that are inhibited by reuterin include species of *Salmonella*, *Shigella*, *Clostridium*, *Staphylococcus*, *Listeria*, *Candida*, and *Trypanosoma* (Daeschel, 1989). Significant interest and research surrounds the application of using reuterin and/or reuterin-producing lactobacilli in the preservation of animal and human foodstuffs by reducing pathogenic and spoilage microorganisms. For example, it was reported that reuterin added to ground

beef inhibits growth of *E. coli* and other contaminating microorganisms on the product (Daeschel, 1989).

Many researchers have reported that LAB produce unique antimicrobial agents that are not acids, bacteriocins or reuterin. Many of these compounds have been investigated although few have been fully characterized. Reports on the existence of other low molecular weight antimicrobial substances produced by LAB are numerous (Fernandes et al., 1987), but to date these substances have neither been identified nor their existence confirmed by other investigations. In two such investigations, lactobacilli of intestinal origin were found to exhibit antimicrobial activity that could not be attributed to either bacteriocins or organic acids (Coconnier et al., 1997; Silva et al., 1987). One example of a newly characterized low molecular weight compound is reutericyclin. It is a tetramic acid derivative produced by sourdough isolates of *Lactobacillus reuteri* (Gänzle, 2000; Höltzel et al., 2000). The inhibitory activity of reutericyclin is limited to Gram-positive bacteria, however the range of antimicrobial activity is broad. Unlike reuterin, yeasts, fungi, and Gram-negative bacteria are resistant to the action of reutericyclin. Research on the production of non-proteinaceous antimicrobial compounds by LAB continues to be investigated and will help further explain the biopreservative role of LAB.

3. MATERIALS AND METHODS

3.1 Challenge Study

a) Bacterial Strains

Bacterial strains used in this study are listed in Table 1. Stock cultures of bacterial strains were maintained at -70°C in APT (All Purpose Tween) broth (Difco Laboratories, Detroit, MI., U.S.A.) or tryptic soy broth (TSB, Difco) with 20% v/v glycerol. The LAB strains used in the applied study were the experimental strains UAL (University of Alberta Lactic Acid Bacteria Collection) 185 and 275 that were designated as a psychrotrophic and bacteriocinogenic strains of *Lactococcus* spp. by Mishra (1992), and *Leuconostoc gelidum* UAL 187. Strains UAL 185 and UAL 275 were morphologically different, hence both strains were included in the study. Subsequent ribotyping results (see 4.2.4) showed similar strain patterns. The *L. monocytogenes* cocktail consisted of *Listeria monocytogenes* List 4, and *Listeria monocytogenes* HPB65 and HPB642. The *L. monocytogenes* cocktail was originally used and characterized by Panayach (1998). The *L. monocytogenes* strains were selected based on studies showing that the strains grew compatibly in the cocktail and could be differentiated by RAPD (random amplification of polymorphic DNA) strain identification technique (Panayach, 1998). Cultures used in the study were prepared by inoculation of frozen cells into APT broth (LAB) at pH 6.6, and TSB broth (*L. monocytogenes*). The strains were subcultured for two consecutive transfers after 18 to 24 hours, at 25°C for UAL 185, UAL 275 and UAL 187 and 37°C for the *L. monocytogenes*.

b) Treatments

Two replicates of the challenge study were conducted on regular beef wieners obtained on two different days of production (Lilydale Foods, Edmonton, Canada). Cultures grown in 10 mL APT broth and TSB broth were prepared for inoculation of the wieners by centrifugation (3500 x *g* for 8 min at 7°C) to harvest the cells (Sorvall® RC-5B refrigerated superspeed centrifuge, Mandell Scientific Co. Ltd.). The cells were washed

Table 1 List of bacterial strains used in the study

STRAIN	REFERENCE NUMBER
Carnobacterium divergens	UAL 9
Carnobacterium piscicola	UAL 8 or LV17
Carnobacterium piscicola	UAL 8C2
Carnobacterium piscicola	UAL 26
Lactococcus lactis	ATCC 11454
Leuconostoc gelidum	UAL 187
Leuconostoc spp.	UAL 280
Listeria monocytogenes	List 4
Listeria monocytogenes	HPB 65
Listeria monocytogenes	HPB 642
Pseudomonas aeruginosa	ATCC 19322
Test strains	UAL 185 and UAL 275

ATCC – American Type Culture Collection

UAL – University of Alberta Lactic Acid Bacteria collection

List 4 – Isolated from pork processing equipment (G. G. Greer, Agriculture and Agri-Food Canada, Lacombe Alberta)

HPB 65 and HPB 642 – Isolated from meat (E. Daley, Health Protection Branch, Health Canada, Ottawa)

three times with sterile 0.85% saline (BDH Chemicals Ltd., Poole, England), to remove any residual bacteriocin from the supernatant. After the final washing the UAL 187, UAL 185, and UAL 275 cells were resuspended in 10 mL of 0.85% saline, and each of the three *L. monocytogenes* strains were resuspended in 300 μL of 0.85% saline. The cultures were thoroughly mixed on a Vortex mixer and added to the corresponding treatments in 4 L of sterile 0.85% saline (stirring), to achieve LAB counts of 10^4 per cm^2 and the *L. monocytogenes* cocktail at 10^3 per cm^2. The wieners were dipped in the treatment solutions for 1 min and dried on a sterile rack. The treatments were as follows:

Treatment #1: sterile 0.85% saline (control sample)
Treatment #2: *L. monocytogenes* cocktail (300 μL of each of the three *L. monocytogenes* strains)
Treatment #3: *L. monocytogenes* cocktail plus 10 mL of UAL 185
Treatment #4: *L. monocytogenes* cocktail plus 10 mL of UAL 275
Treatment #5: 10 mL of UAL 185
Treatment #6: 10 mL of UAL 275
Treatment #7: 10 mL of UAL 187

c) Packaging and Storage

The dried wieners (2 wieners per bag) were aseptically transferred to high barrier film bags (mylar/PVDC/polyethylene vinyl alcohol film, oxygen transmission rate of 7.7 $cc/m^2/24h$; Packall Packaging Inc.) and vacuum packaged (CVP Fresh Vac Model A-300; CVP Systems Inc., Downers Grove, Illinois). All samples were stored at 4°C and the temperature was monitored by a Delphi Temperature Logger (Model 861, New Zealand).

d) Sampling and Plating

Bacteriological sampling was started on day 0 (after inoculation and packaging) and repeated every 7 days to day 56 (8 weeks) and thereafter every 14 days (weeks 10, 12, and 14). The bags were opened with a flame-sterilized scalpel and samples of wiener (1.2 cm in length, approximately 10 cm^2 surface area) were excised with flame-sterilized

scalpels. The sample taken from each of the two wieners in the bag were placed in a sterile Stomacher bag (Seward 400, VWR International, Ltd.) and blended with 90 mL of sterile 0.1% peptone water in a Stomacher (Lab Blender 400) for 2 min. Appropriate serial dilutions in 0.1% peptone water were streaked onto prepoured APT agar (Difco) and PALCAM agar (OXOID Ltd., Basingstoke, Hampshire, England) plates. APT plates were incubated at 25°C for 48 h in anaerobic jars (BBL Anaerobic System; Becton and Dickinson Co., Cockeysville, MD, USA) filled with a gas mixture containing 10% CO_2 and 90% N_2. PALCAM plates were incubated aerobically at 37°C for 48 hours. All colonies were counted on the APT plates while only the grey colonies with a black circumference were counted on the PALCAM plates. Duplicate counts (log cfu/cm^2) were obtained for each sample because both wieners in the bag were sampled separately. The pH of the blended wiener samples was determined by a Fisher Accumet® pH meter 915 (Fisher Scientific Inc., Canada).

3.2 Growth Studies and Inhibitory Activity

3.2.1 Growth Characteristics

Growth of the test culture UAL 185 was determined in Lactobacilli MRS and APT broth (Difco), at 1, 4, 7, 10, and 25°C, based on visible change in culture density. The 1°C and 4°C incubations were done in Lauda Refrigerating Circulators, Model RMS-6 (Brinkmann Instruments Inc., Mississauga, Canada). Samples were taken from the 1°C incubation to determine cell numbers at Day 0, 7, 14, 21, and 28 and plated on Lactobacilli MRS and APT agar (Difco). At all temperatures the strains were inoculated with a 1% inoculum, incubated at a specific temperature and the tubes were visually examined for growth for up to 3 weeks. Strains were used in this study as positive and negative controls were *Carnobacterium divergens* UAL 9, *Carnobacterium piscicola* UAL 8C2, *Lactococcus lactis* ATCC 11454, *Leuconostoc* spp. UAL 280, and *Leuconostoc gelidum* UAL 187. A growth curve for UAL 185 in MRS broth (Difco) at 25°C was obtained by sampling at hourly intervals for 24 hours.

3.2.2 Assays for Inhibitory Activity

The activity spectrum of UAL 185 was assessed by the deferred antagonism method described by Barefoot and Klaenhammer (1983). Both APT and MRS agar plates (1.5% agar) were used and they were inoculated using a Cathra replicating inoculator (KVL Laboratories, Cambridge, Ontario, Canada). The plates were incubated overnight at 25°C under aerobic or anaerobic conditions (BBL Anaerobic System with 5 to 10% CO_2; Becton and Dickinson Co., Cockeysville, MD, USA). After incubation the plates were overlayered with 6 mL of soft (0.75% agar) APT or tryptic soy agar (TSA, Difco) containing 1% inoculum (approximately 10^7 cfu/mL) of an indicator organism. The overlayered plates were incubated under optimum growth conditions for the indicator organisms. After 24 hours zones of inhibition surrounding the producer strain were recorded as inhibition of the indicator organisms. A clear zone of inhibition around the producer organism (10 mm in diameter) was recorded as positive for production of inhibitory substance. Inhibitory activity was also tested by spot-on-lawn assay (Ahn and Stiles, 1990). A 10 µL spot of undiluted supernatant was spotted onto agar plates that were freshly overlayed with an indicator lawn, and incubated for 24 hours to allow for growth of the indicator and appearance of inhibition. The indicator organism used in the spot-on-lawn assay was *C. piscicola* UAL 9.

a) Bacterial Cultures

Stock cultures, maintained at -70°C in APT broth or tryptic soy broth (TSB) with 20% v/v glycerol, were propagated twice (1% inoculum) in fresh APT or TSB prior to use in experiments. Strains were subcultured a maximum of five times before fresh working cultures were prepared from the frozen stock cultures.

The producer organisms used in the deferred inhibition assays were *Lactococcus lactis* ATCC 11454 and *Leuconostoc gelidum* UAL 187 as positive controls, *Carnobacterium piscicola* UAL 8C2 as a bacteriocin-negative control, the experimental strains UAL 185, and UAL 275, a single colony isolated from a pure culture of UAL 185 that displayed a difference in colony morphology.

The indicator organisms selected for the assay included a broad spectrum of pathogenic and non-pathogenic gram positive bacteria. They included *Clostridium butyricum* ATCC 8260, *Staphylococcus aureus* S13, *Staphylococcus epidermidis* ATCC 14990, *Streptococcus bovis* ATCC 15351, two strains of vancomycin resistant *Enterococcus faecium* (VRE) - isolate #R846 from a blood culture and #S769 from midstream urine sample from the Provincial Laboratory, University of Alberta Hospital, two strains of methicillin resistant *Staphylococcus aureus* (MRSA) – isolate #R948 from a nose swab and #R1230 from a abscess swab sample from the Provincial Laboratories, University of Alberta Hospital, *Enterococcus durans* ATCC 19432, *Enterococcus faecalis* isolate #BC 13047, Prov. Lab, U of A Hospital, *Enterococcus faecium* isolate #BC 15537, Provincial Laboratories, University of Alberta Hospital, *Bacillus cereus* ATCC 14579, *Brochothrix campestris* ATCC 43754, *Brochothrix thermosphacta* ATCC 11509, *Listeria monocytogenes* Scott A, *Listeria monocytogenes* List 4, *Listeria monocytogenes* HPB 65 and 642, *Carnobacterium divergens* UAL 9, *Leuconostoc* spp. UAL 280, and *Leuconostoc gelidum* UAL 187.

b) Enzymatic sensitivity

The possible proteinaceous nature of the inhibitory compounds was tested by enzyme sensitivity tests. The enzymes used in the assay were Pronase E from *Streptomyces griseus*, Type XIV (Sigma Chemical Co. St. Louis, MO., U.S.A.), Proteinase K, Fungal (GibcoBRL® Life Technologies, Gaithersburg, MD, U.S.A.), Trypsin from porcine pancreas (Sigma), and α-chymotrypsin from bovine pancreas, Type II (Sigma). A 5μL sample of each enzyme at a concentration of 10 mg/mL was spotted close to the fully grown producer organism on MRS and APT agar plates in a deferred inhibition assay. The enzyme spots was dried for 20 min and overlayered with 6 mL of soft (0.75% agar) APT containing 1% inoculum (approximately 10^7 cfu/mL) of UAL 9 or UAL 8C2 as indicator organisms. The controls used in the assay were *L. lactis* ATCC 11454, producing nisin, *L. gelidum* UAL 187, producing leucocin A, *C. piscicola* UAL 8, producing carnobacteriocins, and *C. piscicola* UAL 8C2, a bacteriocin-negative variant of UAL 8.

3.3 Strain Identification

3.3.1 Physiological and Biochemical tests

a) Cell morphology and gram reaction

Examination of cell morphology and gram reaction was done on colonies picked from an overnight culture streaked on Lactobacilli MRS agar (Difco). A smear of culture was suspended in 100 µL of sterile Milli-Q water and centrifuged at 8,000 x *g* for 5 min. The water was removed and the cells were streaked onto microscope slides and mixed with 2 drops of sterile Milli-Q water. The cell solution was then air-dried and heat fixed onto the slides. The Gram reactions were performed using solutions from a Gram Staining kit (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), and observed with a light microscope at 100X magnification.

b) Catalase reaction

Overnight streaked cultures grown on MRS agar at 25°C were used to determine the catalase reaction. Colonies were obtained from the plates with a sterile loop and inoculated into a 20% v/v hydrogen peroxide solution. A strain of *Pseudomonas aeruginosa* ATCC 19322 was used as a positive control in the assay.

c) Homofermentative versus Heterofermentative metabolism

Differentiation of homofermentative and heterofermentative strains in the study was assayed based on a method described by Zuniga *et al.* (1993). The components of the differential medium (M5) used in the assay were: tryptone (10 g/L), yeast extract (5 g/L), fructose (2.5 g/L), KH_2PO_4 (2.5 g/L), Tween 80 (1 mL/L), L-cysteine HCl (0.5 g/L), $MgSO_4 \cdot 7H_20$ (0.2g/L), $MnSO_4 \cdot H_2O$ (0.05 g/L), calcium pantothenate (0.01 g/L), bromocresol green (20 mL), agar (20 g/L); pH 6.5. The cells were grown until early stationary phase in APT or MRS medium, harvested by centrifugation, and washed twice with sterile Milli-Q water. The pellet was then resuspended in sterile Milli-Q water and appropriate dilutions were spread plated onto the sterilized M5 differential medium.

Plates were incubated anaerobically at 25°C for as long as it was necessary to see colony formation on the plates. Colonies were observed and colors were recorded based on the fact that homofermentative LAB colonies were blue on the M5 medium, whereas heterofermentative LAB colonies remained white.

d) Carbohydrate fermentation

The carbohydrate fermentation patterns of UAL 185, *L. lactis* ATCC 11454, *L. gelidum* UAL 187, and *Leuconostoc* spp. UAL 280 were determined twice by API 50 CHL identification strips (bioMerieux Vitek Inc., France). Overnight cultures of LAB were prepared for strip inoculation according to the manufacturer's instructions. Colonies were selected from the MRS agar plates and suspended in 2 mL of sterile Milli-Q water. This cell suspension was used to inoculate 5 mL of sterile Milli-Q water until an OD_{600} of approx 0.35 was achieved with a Spectronic 21 spectrophotometer (Bausch & Lomb, USA). The number of drops of cells used to reach the desired OD_{600} was doubled and this amount was added to 10 mL of CHL medium (bioMerieux). The inoculated CHL medium was then used to inoculate the test API strips. The inoculated test strips were incubated at 25°C and color changes were recorded after 3, 6, 24 and 48 hours of incubation. Specific carbohydrate fermentation patterns for the strains were submitted to API (bioMerieux Canada, Inc., St-Laurent, Quebec). Resulting matches of the fermentation profile of the experimental strain to the existing profiles in the API database were obtained.

3.3.2 Ribotyping

The experimental strains UAL 185 and UAL 275 and the reference strain *L. lactis* ATCC 11454 were inoculated into 1 mL of soft APT (0.75%) agar and sent to the University of Guelph (Canadian Institute of Food Safety Research, Guelph Ontario) for ribotyping. Ribotype patterns were obtained from the automated RiboPrinter® Microbial Characterization System (Qualicon Inc., Wilmington, Del.©).

3.3.3 16S rDNA Sequencing

a) Chromosomal DNA Isolation

Chromosomal DNA was extracted from UAL 185 based on the chromosomal DNA isolation protocol (Quadri et al., 1994). The strain was grown overnight in 40 mL of APT broth at 25°C. The cells were harvested by centrifugation at 9,000 x *g* for 15 min. The cell pellet was washed twice with 20 mL of cold 0.5% NaCl and centrifuged between and after washings at 9,000 x *g* for 20 min and 10,000 x *g* for 20 min, respectively. The washed cells were resuspended in 3 mL of solution A (62.5 g sucrose, 12.5 mL 1 M Tris, pH 8.0, and 2.5 mL 0.5 M EDTA, pH 8.0), adjusted to pH 8.0 with 10 N NaOH. Lysozyme was added to the sample to give a concentration of 20 mg/mL, and the mixture was incubated at 37°C for 1 hour with intermittent shaking. After incubation, 3 mL of lysis buffer (1% SDS and 100 mM EDTA, pH 8.0), preheated to 65°C was added to the mixture at 65°C, and held for 15 min. Proteinase K solution was added at a final concentration of 100 µg/mL and incubated at a maximum temperature of 56°C for approximately 6 h. A volume of 0.6 mL, 1 M NaCl was added prior to the phenol/chloroform precipitation. An equal volume of phenol was added and mixed gently, followed by the addition of an equal volume of chloroform and mixed by gentle inversion. The resulting mixture was centrifuged at 12,000 x *g* for 20 min and the aqueous phase was removed for two more phenol/chloroform precipitations to ensure that the aqueous phase was clear. After the three consecutive precipitations 1 volume of chloroform:isoamyl alcohol (24:1) was added and mixed gently by inversion, to remove any residual phenol. Centrifugation at 10,000 x *g* for 10 min was performed prior to removing the aqueous phase containing the DNA. A 1/10 volume of 3 M sodium acetate (pH 5.2) and 2X volume of 95% DNA grade ethanol was added to the aqueous phase, and the mixture was held for 1 hour at -20°C. After the ethanol precipitation, the mixture was centrifuged at 12,000 x *g* for 20 min and the resulting DNA pellet was washed twice with 70% ethanol, centrifuged initially at 12,000 x *g* for 20 min and at 10,000 x *g* for 2 min. The remaining ethanol was evaporated under vacuum (SpeedVac SC100, Savant Instruments Inc., England) until the pellet was completely dry. The dried DNA pellet

was dissolved in 50 μL of sterile Milli-Q water, and stored at -20°C for further analysis by agarose gel electrophoresis and used in the PCR amplification of bacterial 16S rDNA.

b) PCR Amplification of Bacterial 16S rDNA

Chromosomal DNA manipulation was done based on a method published by Wilson et al., (1990). The extracted nucleic acids from the chromosomal DNA isolation were suspended in 50 μL of Milli-Q water. The 10X PCR buffer (100 mM Tris, pH 8.3, 500 mM KCl, 9 mM $MgCl_2$, 0.1% gelatin) was added to the PCR tubes in 10 μL volumes. Oligonucleotide primers (synthesized by University of Alberta, DNA Laboratory) were specified, with modifications to anneal to priming sites at the extreme 5' end, the extreme 3' end, and the center of 16S ribosomal DNA.

Primers PC3mod (5'-GGACTAHAGGGTATCTAAT-3'), POmod (5'-AGAGTTTG ATCMTGG-3'), PC5 (5'-TACCTTGTTACGACTT-3'), and P3mod (5'-ATTAG ATACCCTDTAGTCC-3') were added to the PCR reactions at a final concentration of 1 μM. The dNTPs were added at a final concentration of 200 μM and sterile Milli-Q water was added to the reaction mixture to bring the final volume to 100 μL. Immeadiately prior to starting the PCR reaction, 2.5 units of *Taq* polymerase (GibcoBRL®,) was added to each reaction tube and mixed gently. The specific primers required a modification to the standard PCR protocol. PCR reactions were run on the GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT). The annealing temperature was changed from 55°C to 33°C for 30 s, and the extension phase from 72°C for 3 min to 75°C for 4 min. The denaturing step was done at 90°C for 70 s, followed by the modified annealing and extension profiles. The protocol specified for 2 cycles of denaturing at 94°C for 45 s, followed by 25 cycles of the above-mentioned denaturing, annealing and extension cycles. The reaction tubes were incubated for an additional 10 min at 72°C and thereafter the reaction mixture was maintained at 4°C. Blank control tubes (without added nucleic acids) were run in the experiment to control for inadvertent introduction of exogenous nucleic acids. PCR product samples were stored at -20°C until they were analyzed by agarose gel electrophoresis. The PCR products (10 μL) were analyzed on gels prepared

using 1% w/v agarose (Life Technologies, Inc. Gathersburg, MD, USA) and 5 μL ethidium bromide (10 mg/mL) stain (BioRad Laboratories, Hercules, CA, USA) in 100 mL of TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). A 100 kb DNA ladder (GibcoBRL®) was run on the gel as a molecular weight standard to check for the correct size of the amplified DNA. The gels were submerged in TAE buffer in a gel electrophoresis unit (Tyler Research Instruments, Canada), loaded with samples (mixed with 2 μL loading dye), and subjected to a constant voltage of 100 volts supplied by an Electrophoresis Power Supply (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.), for 1 to 2 hours. Visualization of the bands under UV light was performed using either the UV light box (Fotodyne Inc., New Berlin, WI, USA) or a Gel Doc 1000 and accompanying Molecular Analyst software (Bio-Rad).

c) Sequencing of Amplified 16S rDNA

The amplified segments of 16S rDNA from UAL 185 were purified with QIAquick spin columns in a microcentrifuge (QIAquick PCR purification kit protocol). After purification and assessment that the DNA was present in sufficient concentration and of the expected sizes, the PCR products were sent for DNA sequencing where it was sequenced directly using a CEQ dye terminator cycle sequencing kit (Beckman Coulter Inc., Fullerton, CA) on a CEQ 2000 sequencer (Department of Biochemistry, University of Alberta) with the corresponding primers. The sequences generated for the two fragments were used in homology comparisons with the BLAST network service at the National Center for Biotechnology Information (NCBI).

3.4 Plasmid Studies

3.4.1 Plasmid Curing and Variant Screening

Curing the plasmid(s) of UAL 185 was based on a procedure used by Hastings for his work on a bacteriocin produced by *Leuconostoc gelidum* (PhD thesis, 1991). Novobiocin (Sigma) was diluted in Lactobacilli MRS broth containing 0.0002% sodium dodecyl sulphate (SDS) to give final concentrations' of 25 to 50 μg/mL at 5 μg/mL

intervals. The inoculum was approximately 1 x 10^5 cells of UAL 185 per mL and the cultures were incubated at 25°C prior to dilutions and assays for the antimicrobial activity.

3.4.2 Small-Scale Plasmid Isolation

Plasmid DNA was extracted using the alkaline method for plasmid DNA isolation (Birnboim and Doly, 1979), with modifications (van Belkum, University of Alberta laboratory protocol). The strains used in the small-scale plasmid isolation procedure were UAL 185, *L. lactis* ATCC 11454, *C. piscicola* UAL 8, *L. gelidum* UAL 187, and *C. piscicola* UAL 26. A 1.5 mL sample of overnight culture was harvested by centrifugation at 16,000 x *g* for 3 min (Eppendorf Microcentrifuge 5417C, Brinkmann Instruments Inc., Mississauga, Canada). The supernatant was discarded and the cell pellet was washed with 100 µL sterile Milli-Q water. The cell pellet was redissolved in 200 µL TE buffer (50 mM Tris-Cl; 10 mM EDTA) with lysozyme (Sigma) added to give a concentration of 10 mg/mL. The mixture was incubated at 37°C for 90 min. After incubation, 200 µL of lysis buffer (1% SDS, 0.2 N NaOH) was added and inverted until the solution cleared. Directly after addition of the lysis buffer, 200 µL of cold 3 M potassium acetate was added. The solution was centrifuged at 14,000 rpm for 10 min. The resulting aqueous phase was added to a mixture of 400µL phenol and 400 µL chloroform. The mixture was mixed on a Vortex mixer for 10 seconds and centrifuged at 16,000 x *g* for 6 min. The upper phase was extracted and added to 400 µL chloroform:isoamyl alcohol (24:1). The mixture was mixed using a Vortex mixer for 10 seconds and centrifuged at 16,000 x *g* for 6 min. The upper phase was added to 800 µL of 95% DNA grade ethanol and stored at -20°C overnight. After overnight precipitation, the ethanol solutions were centrifuged at 16,000 x *g* for 15 min. The ethanol was removed and the pellet was washed twice with 500 µL 70% DNA grade ethanol. The remaining ethanol was evaporated under vacuum (SpeedVac SC100) until the pellet was completely dry. The dried DNA pellet was dissolved in 10 µL of TER (50 mM Tris, 10mM EDTA solution with 20 µg/mL ribonuclease A) buffer and incubated at 37°C for 1 hour. The 10 µL DNA samples were loaded onto a 1% agarose gel and subjected to a

constant voltage of 90 volts for 2 hours. Plasmid profiles were visualized under UV light using a Gel Doc 1000, with Molecular Analyst software (BioRad). A lambda DNA ladder cut with *Eco*RI and *Hin*dIII restriction enzymes was run with the samples.

3.4.3 Large-scale Plasmid Isolation

The UAL 185 strain was grown in 200 mL MRS broth for 24 hours and the cells were harvested by centrifugation at 8,000 x *g* for 6 min. The supernatant was discarded and the cells were washed in 20 mL STE (TE buffer and 0.5% NaCl) and centrifuged at 8,000 x *g* for 10 min. The washed cells were resuspended in 8 mL of alkali lysis solution I (20 mL Milli-Q water, 5 g sucrose, 400 µL of 0.5M EDTA, 200 µL of 1.0 M Tris, 1 mL of 1.0 M NaCl), mixed well, and after resuspension 10 mg of lysozyme (Sigma) was added per mL and 100 µL of 2,000 U mutanolysin (Sigma) were added. The treated cells were incubated for 1 h at 37°C. After incubation 16 mL of fresh lysis solution II (12 mL of 10% SDS, 0.8 mL of 10 M NaOH, 27.2 mL of Milli-Q water) was added, mixed until the solution cleared and, within 5 min, 12 mL of ice cold alkali lysis solution III (60 mL of 5 M potassium acetate, 11.5 mL glacial acetic acid, 28.5 mL Milli-Q water) was added. The resulting solution was mixed well and kept on ice for 5 to 10 min. The solution was centrifuged for 15 min at 8,000 x *g* and the supernatant was gently transferred to sterile centrifuge tubes. Phenol/chloroform precipitation of DNA was performed (1 mL of phenol:5 mL of chloroform/tube) and centrifuged at 15,000 x *g* for 10 min. The supernatant was divided into two tubes (15 mL in each tube) and 15 mL of isopropanol was added and held at room temperature for 10 min to precipitate the DNA. After precipitation, the mixture was centrifuged at 15,000 x *g* for 15 min and the supernatant was discarded. The pellet was washed with 10 mL 70% ethanol, centrifuged at 15,000 x *g* for 10 min and dried for 5 min under vacuum (SpeedVac SC100). The dried pellet was dissolved completely in 6 mL of TE buffer with 6 g of added cesium chloride. The solution was added to centrifuge tubes containing 300 µL of ethidium bromide (final concentration 10 mg/mL) and centrifuged at 49,000 x *g* for 20 h at 20°C. The lower plasmid band was removed under transillumination. The ethidium bromide was extracted by mixing with isoamyl alcohol. The DNA was transferred to dialysis tubing and

dialysed against TE buffer for 3 h. After dialysis a phenol/chloroform extraction was done and the DNA was precipitated with 40 µL of sodium acetate and 900 µL of 95% ethanol. Washing with 700 µL of 70% ethanol was repeated twice and the pellet was dried under vacuum (Speed-Vac) for 5 min. The dried DNA pellet was dissolved in 40 µL of TE buffer and loaded onto a 1.5% agarose gel for electrophoresis.

3.5 Concentration of Antimicrobial Sunstance(s)

3.5.1 XAD-8 Hydrophobic Exchange Column

An inoculum of 50 µL (0.1%) of overnight culture of UAL 185, *L. lactis* ATCC 11454, and *L. gelidum* UAL 187 was added to 50 mL of MRS (UAL 185) or 50 mL of APT (ATCC 11454 and UAL 187) broth. Supernatants were obtained by centrifugation at 8,000 x *g* for 10 min at 4°C (Sorvall® RC-5B refrigerated superspeed centrifuge, Mandell Scientific Co. Ltd.). Samples of unconcentrated supernatant were collected and heat treated at 100°C for 3 min for activity testing. Supernatant samples were loaded onto a 6 x 25 cm Amberlite XAD-8 column (Pharmacia, Uppsala, Sweden). The column was washed with 0.1% TFA (trifluoroacetic acid) (1.5:1), 40% ethanol/0.1% TFA (1:1), 70% ethanol/0.1% TFA (1:1), and 95% ethanol/0.1% TFA (1:1). Samples of each of the fractions were collected, kept frozen at -20°C, and assayed for inhibitory activity by spot-on-lawn assays against *C. divergens* UAL 9 indicator. The active fractions were dried by rotary evaporation.

3.5.2 Butanol Extraction

A 2 L culture of UAL 185 grown in MRS broth and a 2 L culture of the control strain, *Brochothrix campestris* ATCC 43754, producing brochocin-C was used for the butanol extraction. The overnight culture was centrifuged at 8,000 x *g* for 10 min at 4°C and the supernatant was removed and added to a 4 L Erlenmeyer flask. Butanol (700 mL) was added to the supernatant and mixed for 1 min. The mixture was centrifuged at 6,000 x *g* for 10 min and the upper phase was transferred to a 2 L flask. Butanol (500 mL) was

added and the procedure was repeated. The final butanol extraction consisted of 300 mL of butanol added to the upper phase from the previous steps where it was again mixed and centrifuged. The upper phase was carefully removed and added to a 1 L evaporating flask that was placed on the high-vacuum rotary evaporater. A 1 mL fraction was removed to test for activity in the unevaporated butanol fraction. The butanol solvent was completely removed by rotary evaporation and the remaining solid extract was redissolved in 20 mL of sterile Milli-Q water. This fraction was stored at -20°C until it was used to test for activity by spot-on-lawn assay against the indicator organism, *Carnobacterium divergens* UAL 9.

4. RESULTS

4.1 Challenge Study

The LAB and the *L. monocytogenes* cocktail were inoculated onto the surface of the wieners by dipping them into a saline solution containing 10^5 LAB and 10^4 *L. monocytogenes* per mL. The control and inoculated samples were vacuum packaged at room temperature and stored at 4°C. This resulted in the desired 10-fold difference in the mean inoculum levels between LAB and *L. monocytogenes* on the surface of the wieners, in both replicates. Appropriate dilutions of the wiener samples were plated onto APT agar incubated in an anaerobic atmosphere at 25°C to determine the LAB count and onto PALCAM agar incubated aerobically at 37°C to determine the *L. monocytogenes* count. The mean LAB count was 3.89 log cfu/cm^2 and the mean *L. monocytogenes* count was 2.64 log cfu/cm^2. The initial pH of the blended wiener slurries was 6.40 and 6.43 for replicates 1 and 2, respectively. The initial load of adventitious bacteria on the wieners was 2.69 and 1.88 log cfu/cm^2 for replicates 1 and 2, respectively. The only noticeable difference between replicates 1 and 2 was the initial load of adventitious bacteria on the wieners. The storage temperature was monitored with a data logger throughout the 14-week storage period, a mean temperature of 4.29°C was recorded. Based on the lack of temperature change in the data logger interrogation report it was apparent that the defrost cycle did not cause a change in the storage temperature of the product.

The data in Figure 1 represent the mean plate counts on APT and PALCAM from the two replicates of the challenge study. The error bars represent the standard deviation between replicate data sets. The experimental strains UAL 185 and 275 grew to maximum population on the vacuum packaged wieners within 3 weeks of storage at 4°C and remained at that level for the duration of the study. However, *L. gelidum* UAL 187 grew to maximum population within 6 weeks of incubation, indicating a marked difference between the growth of the experimental and the UAL 187 reference strain on the product. The background microflora on the control sample reached an average of log 7.0 cfu/cm^2 on the wieners, after the final sampling time at 14 weeks of storage.

The data for pH and growth of *L. monocytogenes* on the wieners for replicates 1 and 2 are shown in Figures 2 and 3, respectively. The change in pH of the wieners over time differed between replicates (Figures 2A and 3A). In replicate 1, the pH of the product with added LAB decreased to between pH 4 and 5 over the 14-week storage period. In contrast, sample pH of the uninoculated control, the *L. monocytogenes* control and the sample inoculated with the reference strain UAL 187, decreased at a slower rate. In replicate 2, the difference between the samples inoculated with LAB and the controls was less distinct and the overall decrease in pH was less. However, in Figures 2B and 3B it can be seen that the growth of *L. monocytogenes* was similar between replicates, indicating that the differences in pH between replicates did not influence the inhibitory effect on the *L. monocytogenes*. There was a 4.67 and 5.05 log cfu/cm^2 difference in *L. monocytogenes* count after 14 weeks of storage in replicates 1 and 2, respectively. In replicate 1, the *L. monocytogenes* control grew to a maximum population of 7 to 8 log cfu/cm^2 within 6 weeks of storage. In replicate 2, the pattern of *L. monocytogenes* growth was different. It plateaued at 5 log cfu/cm^2 after 2 weeks and remained at that level until 6 weeks of storage and then increased to between 7 and 8 log cfu/cm^2.

The data for the pH of the meat and the total LAB and *L. monocytogenes* counts were subjected to statistical analysis by ANOVA for each sampling time over the 14 weeks of the study. The means were ranked by Student Newman Keul's (snk) multiple range test (Steele and Torrie, 1980) and are presented in the Appendix (Section 7). Least square means with snk groupings are reported in Table 7.1 for presumptive LAB counts, Table 7.2 for *L. monocytogenes* counts, and Table 7.3 for pH measurements of the replicates. At 14 weeks of storage there was no significant difference among LAB treatments because the adventitious microflora of the control sample had grown to maximum population. The *L. monocytogenes* counts were not significantly different initially (Day 0), but thereafter the *L. monocytogenes* in the presence of UAL 185 and UAL 275 were significantly lower than the number of *L. monocytogenes* on the control samples.

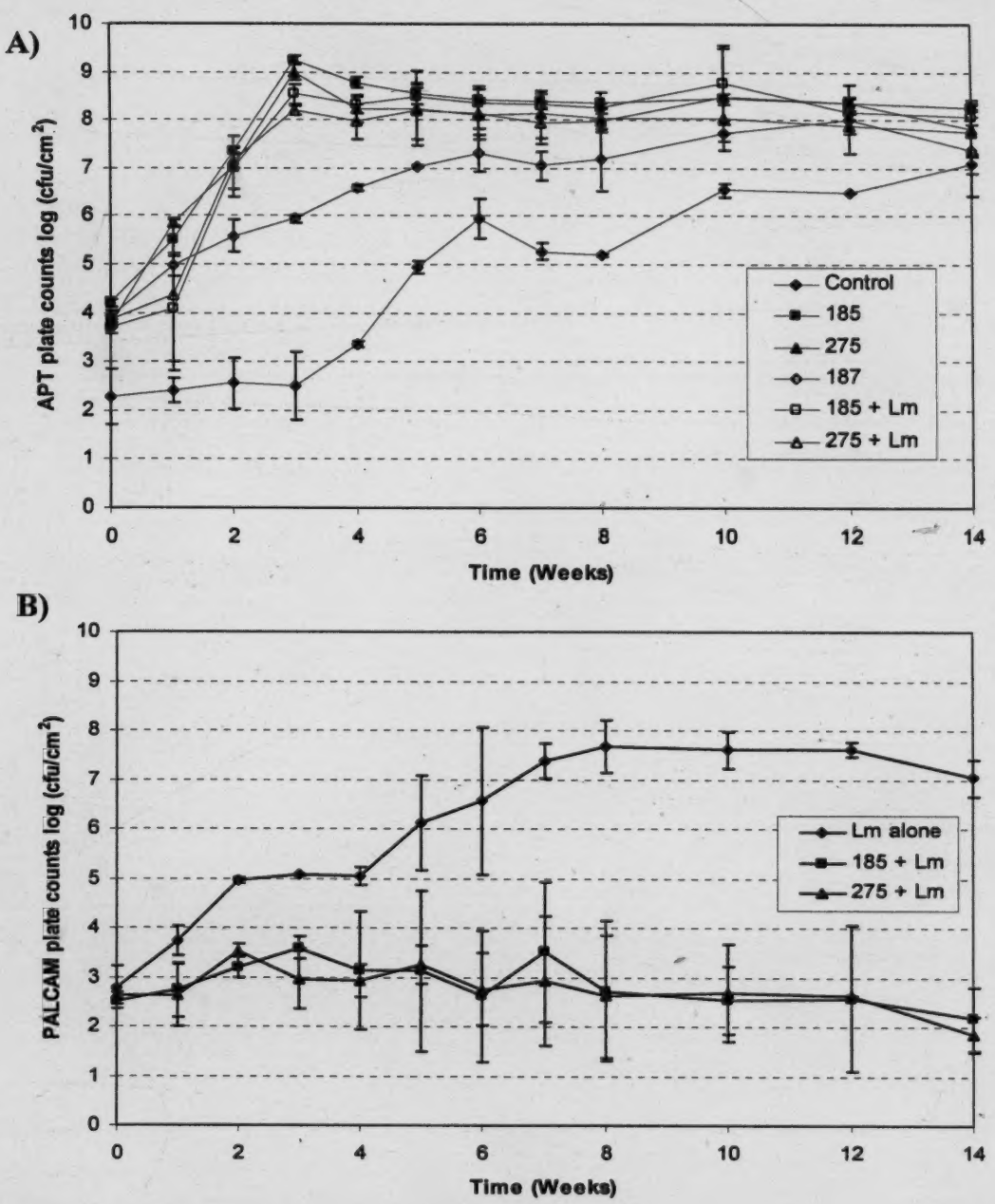


Figure 1 Plate counts represented by mean (log cfu/cm^2) of two samples from two replicates (error bars represent standard deviation of counts between the replicates)

A) Growth of LAB on APT

B) Growth of *L. monocytogenes* on PALCAM

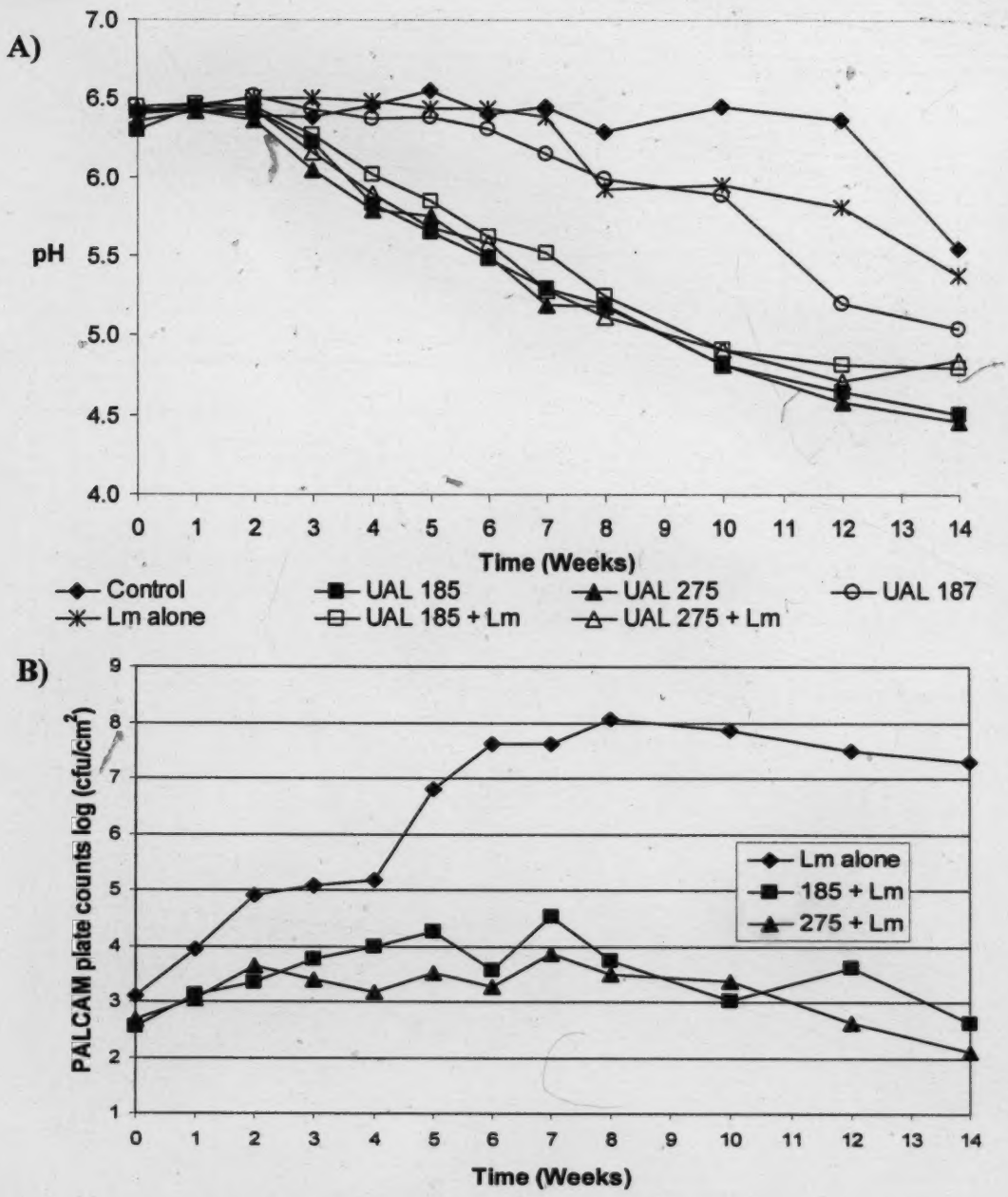


Figure 2 The pH and growth of *L. monocytogenes* on vacuum packaged wieners prepared for replicate 1 and stored at 4°C for 14 weeks

A) pH measurements of blended samples

B) *L. monocytogenes* counts on PALCAM plates

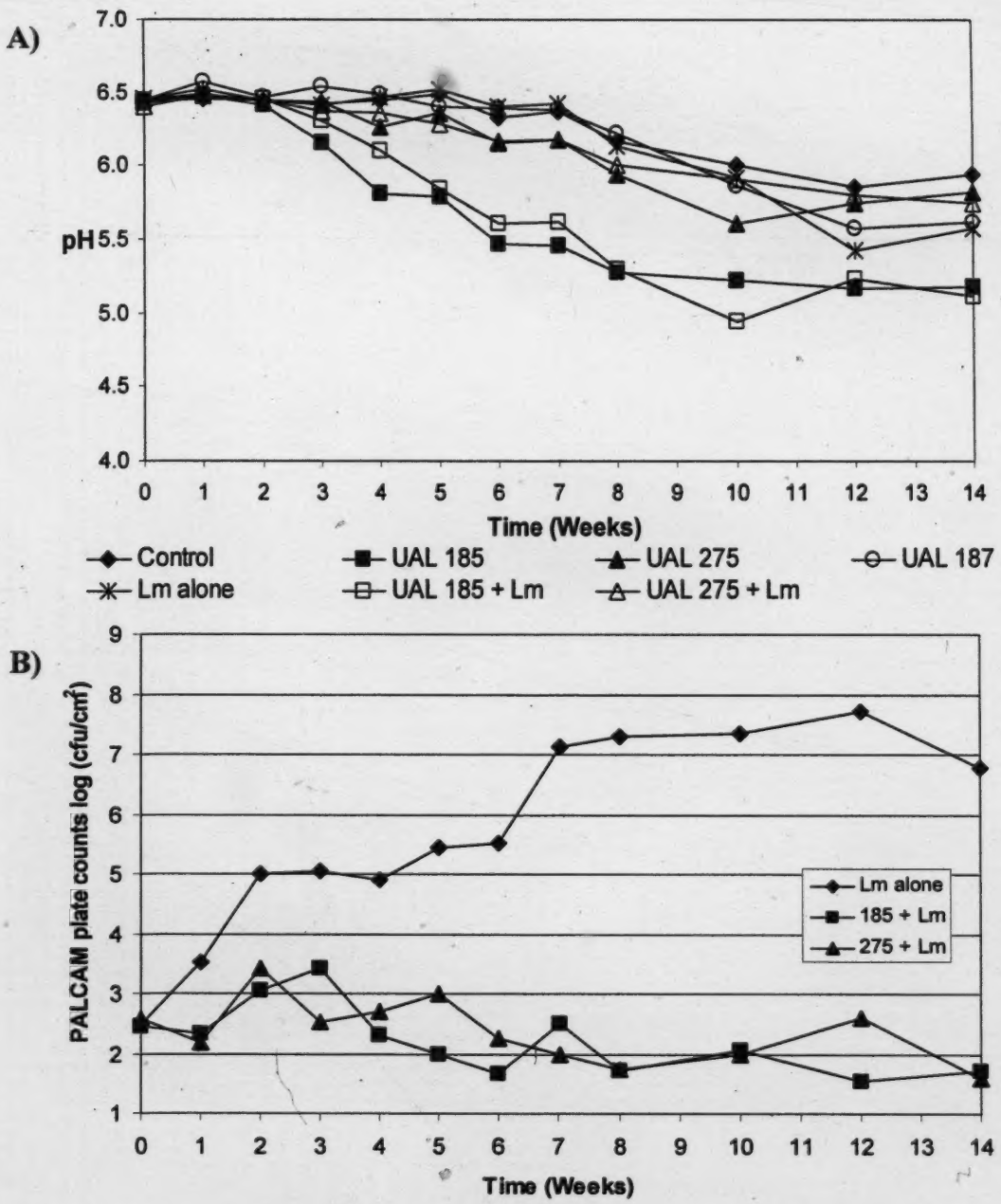


Figure 3 The pH and growth of *L. monocytogenes* on vacuum packaged wieners prepared for replicate 2 and stored at 4°C for 14 weeks

A) pH measurements of blended samples

B) *L. monocytogenes* counts on PALCAM plates

Due to the large difference in pH data between replicates the standard errors of the means (SEM) were large resulting in no significant differences between treatments, except at 4 and 6 week sampling times. Consequently the data for each replicate are presented separately in the results (See Figures 2 and 3).

4.2 Growth Studies and Inhibitory Activity

4.2.1 Growth Characteristics

Growth of 1% inoculum of the test strains in broth, at different temperatures, based on evaluation of turbidity is shown in Table 2. As expected, all strains except *L. lactis* ATCC 11454 grew at 4°C. The *Leuconostoc* strains grew slower at 4°C than the strains of *Carnobacterium* spp. and the experimental UAL 185 strain. All of the psychrotrophic LAB strains in the study grew to maximum cell density based on culture turbidity within 48 hours at 7°C. All of the strains were fully grown within 24 hours of incubation in broth at 25°C.

Data for the growth of UAL 185, UAL 275, and ATCC 11454 shown in Figure 4 were determined by plating from MRS and APT broth cultures incubated at 1°C. The experimental strains grew to maximum population within 14 days. The reference strain *L. lactis* ATCC 11454 did not grow at 1°C. Growth data at 1°C were also obtained for *C. divergens* UAL 9, *C. piscicola* UAL 8C2, *L. gelidum* UAL 187 and *L. gelidum* UAL 280. These data are not presented in Figure 4; however, the growth of these psychrotrophic LAB was comparable to that of the test strains UAL 185 and 275.

Growth curves for a 0.01% inoculum of UAL 185 in MRS broth at 25°C are shown in Figure 5. The data include measurements of: pH during growth; absorbance change at OD_{650}; and bacterial numbers determined by aerobic plate counts on MRS agar incubated at 25°C. At 25°C UAL 185 grew to a maximum population of 9 log cfu/mL and the pH of the medium dropped to pH 4.5 within approximately 10 hours of incubation.

Table 2 Time to detection of visible growth of selected lactic acid bacteria (1% inoculum) in APT broth at 4°C, 7°C and 25°C.

STRAINS	TEMPERATURES		
	4°C	7°C	25°C
ATCC 11454	> 200 hours ~	48 hours ~ 72 hours + 96 hours + +	24 hours + +
UAL 185	72 hours + 96 hours ++	24 hours + 48 hours ++	24 hours + +
UAL 275	72 hours + 96 hours ++	24 hours + 48 hours ++	24 hours + +
UAL 9	72 hours + 96 hours ++	24 hours + 48 hours ++	24 hours + +
UAL 8C2	72 hours + 96 hours ++	24 hours + 48 hours ++	24 hours + +
UAL 187	96 hours + 120 hours + +	24 hours ~ 48 hours ++	24 hours + +
UAL 280	96 hours + 120 hours + +	24 hours ~ 48 hours ++	24 hours + +

~ minimum growth; culture turbidity unchanged by visual observations
+ medium growth; noticeable change in turbidity
+ + full growth; maximum cell density observed in broth culture

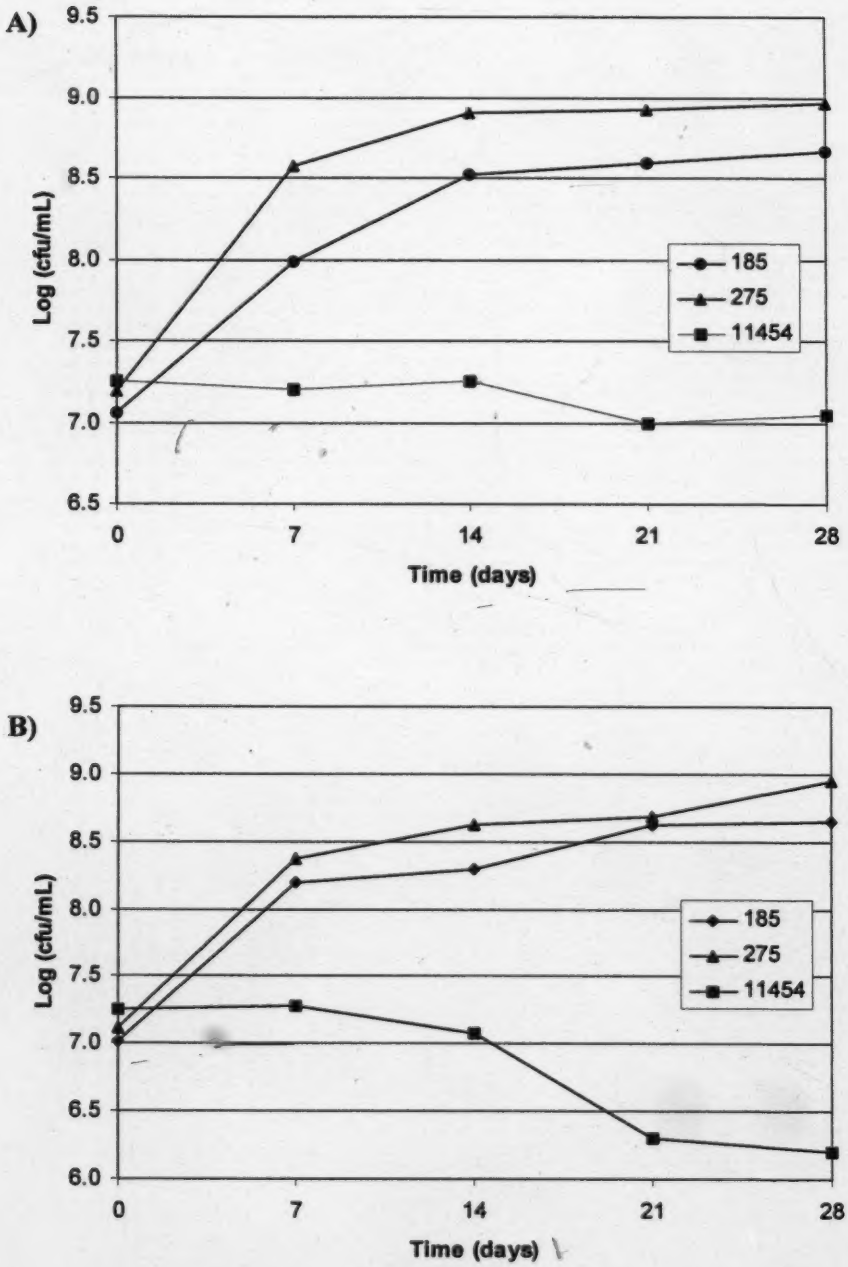


Figure 4 Growth of UAL 185, UAL 275, and *L. lactis* ATCC 11454 at 1°C in: A) APT broth and B) MRS broth.

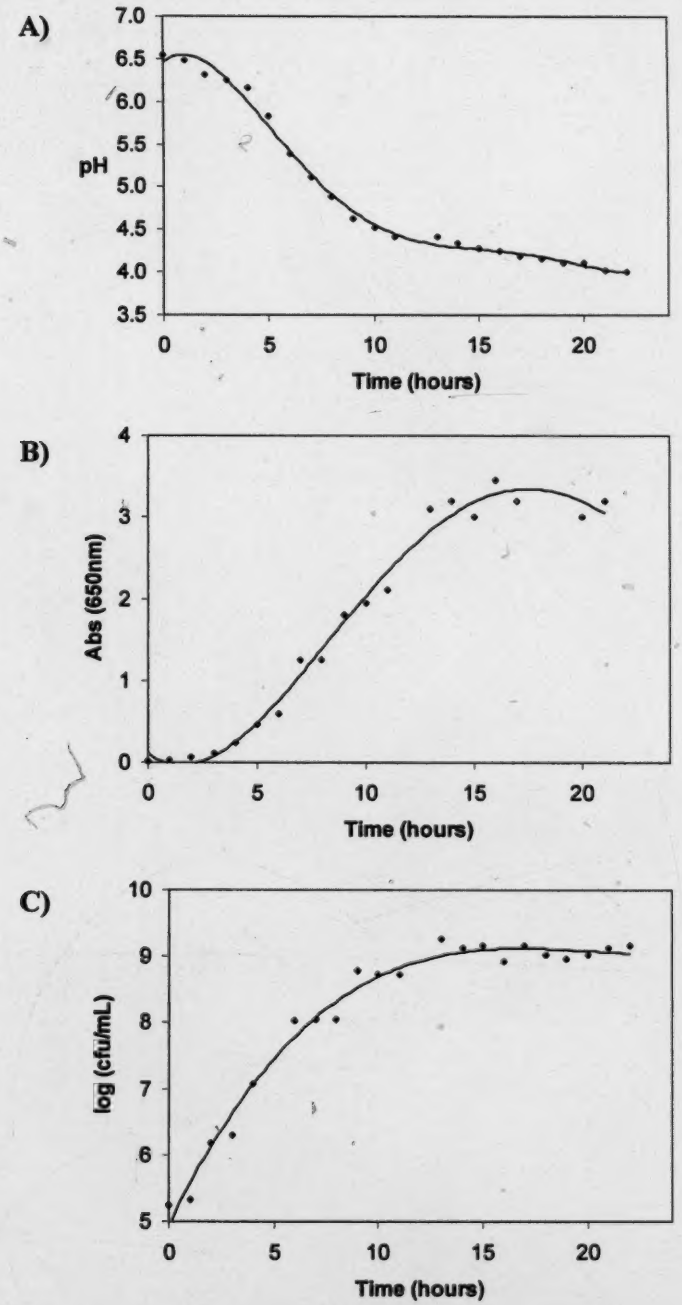


Figure 5 pH change and growth determined by absorbance and plate count of a 0.01% inoculum of UAL 185 in MRS broth at 25°C.

A) pH B) Absorbance (A_{650}) C) log cfu/mL

4.2.2 Assays for Inhibitory Activity

Results of the deferred inhibition assay against a range of indicator organisms are shown in Table 3 and indicate that UAL 185 inhibited a broad spectrum of organisms. This inhibition was noticeably greater when the test strain was grown on MRS agar. There was no major difference between the strains grown under aerobic or anaerobic conditions. When differences were observed, they are represented in Table 3 as two observations, with the first being inhibition with the aerobic growth of the producer organism. The activity range of UAL 185 can be compared with that of nisin produced by *L. lactis* ATCC 11454. Overall, the spectrum of activity was comparable between UAL 185 grown on MRS and ATCC 11454 grown on either medium. Exceptions to this are the inhibition of the indicator *Staphylococcus* spp. MRSA isolate #R948 where UAL 185 was more active than ATCC 11454, and inhibition of the *Leuconostoc* spp. because UAL 185 did not inhibit the strains tested while ATCC 11454 exhibited considerable actimicrobial activity against these strains.

The enzymatic sensitivity of the inhibitory substances was determined in a deferred inhibition test using UAL 9 and UAL 8C2 as indicators. It was concluded that the substance(s) produced by UAL 185 is not of a proteinaceous nature. The enzymes used were active because inactivation of the bacteriocins produced by the reference strains, *L. lactis* ATCC 11454, *C. piscicola* UAL 8, and *L. gelidum* UAL 187 indicated enzymatic sensitivity.

Table 3 Inhibitory activity[1] of selected lactic acid bacteria strains grown on APT and MRS agar against pathogenic and non-pathogenic indicator organisms determined by deferred inhibition tests.

	PRODUCER STRAINS[2]				
INDICATOR STRAINS	**ATCC 11454**	**UAL 185**	**UAL 275**	**UAL 187**	**UAL 280**
Clostridium butyricum ATCC 8260 – APT	++	-	-	+	- +
Clostridium butyricum ATCC 8260 – MRS	+++	+	+	+	-
Staphylococcus aureus S13 – APT	+++	+	+	++	+
Staphylococcus aureus S13 – MRS	+++	++	++~	+++	~
Staphylococcus epidermidis ATCC 14990 – APT	++	-	-	-	+
Staphylococcus epidermidis ATCC 14990 – MRS	++	++	++	-	-
Streptococcus bovis ATCC 15351 – APT	++	-	-	-	-
Streptococcus bovis ATCC 15351 – MRS	++~	++	++	-	-
Enterococcus spp. VRE – Isolate # R846 – APT	++~	-	~	-	~
Enterococcus spp. VRE – Isolate # R846 – MRS	++	++	++	+	+ ++
Enterococcus spp. VRE – Isolate # S769 – APT	++	-	~	-	~
Enterococcus spp. VRE – Isolate # S769 – MRS	++	++	+~	~	~
Staphylococcus spp. MRSA -Isolate # R948 – APT	-	-	-	-	~
Staphylococcus spp. MRSA–Isolate #R948 – MRS	~	++	++	-	+
Staphylococcus spp. MRSA–Isolate#R1230 – APT	+++	-	-	-	~
Staphylococcus spp. MRSA –solate #R1230 - MRS	++~	++	++~	-	- +

INDICATOR ORGANISMS	ATCC 11454	UAL 185	UAL 275	UAL 187	UAL 280
Enterococcus durans ATCC 19432 – APT	++	~	-	+~	-
Enterococcus durans ATCC 19432 – MRS	++	+~	+	++	-
Enterococcus faecalis # BC 13047 – APT	+	~	-	+	-
Enterococcus faecalis # BC 13047 - MRS	+	+	+~	+~	-
Enterococcus faecium #BC 15537 - APT	+~	~	~	+	-
Enterococcus faecium #BC 15537 - MRS	++	+	+	+	-
Brochothrix campestris ATCC 43754 - APT	+++	+	+	+	+
Brochothrix campestris ATCC 43754 - MRS	+++	++	++	+	++
Brochothrix thermosphacta ATCC 11509 – APT	+++	+	~	+	+~
Brochothrix thermosphacta ATCC 11509 – MRS	+++	++	++	+ ++	++
Listeria monocytogenes Scott A – APT	++~	+	+	++	+~
Listeria monocytogenes Scott A – MRS	++~	++	++	++	+
Listeria monocytogenes List 4 – APT	++	~	-	++	-
Listeria monocytogenes List 4 – MRS	++	+	+	++	-
Listeria monocytogenes HPB 65 – APT	++	~	~	++	-
Listeria monocytogenes HPB 65 – MRS	++	++	++	++	+
Listeria monocytogenes HBP 642 – APT	++	~	-	++	-
Listeria monocytogenes HBP 642 - MRS	++	++	++	++	~

INDICATOR ORGANISMS	ATCC 11454	UAL 185	UAL 275	UAL 187	UAL 280
Leuconostoc spp. UAL 280 - APT	+++	-	-	+	-
Leuconostoc spp. UAL 280 - MRS	+++	~	~	+~	-
Leuconostoc gelidum UAL 187 - APT	+++	-	-	-	- +
Leuconostoc gelidum UAL 187 - MRS	+++	-	-	-	~
Carnobacterium divergens UAL 9 – APT	++	~	+	++	+
Carnobacterium divergens UAL 9 – MRS	+~	+~	+~	++	+
Carnobacterium pisicola UAL 8C2 - APT	++	-	~	+~	+
Carnobacterium pisicola UAL 8C2 - MRS	++	++	++	++	+~

[1]

-	no inhibitory activity
~	< 1 cm diameter zone of inhibition
+	1 cm diameter zone of inhibition
++	2 cm diameter zone of inhibition
+++	3 cm diameter zone of inhibition

[2] *L. lactis* ATCC 11454

Test strain UAL 185

Test strain UAL 275

L. gelidum UAL 187

Leuconostoc spp. UAL 280

4.3 Strain Identification

4.3.1 Physiological and Biochemical tests

The test strains, UAL 185 and 275 were blunt rod-shaped Gram-positive organisms and not coccus-shaped as expected. Both strains were catalase-negative and heterofermentative.

The fermentation profiles determined by API 50CH test strips were obtained for strains UAL 185 and 275 and indicated minor differences between these strains. The fermentation profiles for UAL 185 and three reference strains are shown in Table 4. The principal reference strain was *L. lactis* ATCC 11454. In addition *L. gelidum* UAL 187 and *Leuconostoc* spp. UAL 280 were included for comparison purposes. The facsimile results based on the bioMerieux database, classified the fermentation pattern for UAL 185 as having the greatest homology to *Lactococcus lactis*. The reference organism ATCC 11454 was correctly classified as *L. lactis*. *L. gelidum* UAL 187 and *Leuconostoc* spp. UAL 280 were both classified as *Leuconostoc mesenteroides*.

4.3.2 Ribotyping

Strains UAL 185, 275 and *L. lactis* ATCC 11454 were submitted to the University of Guelph Ribotyping Center for strain identification on their Riboprinter® Microbial Characterization System (Qualicon, Inc.). The report (Figure 6A) indicated that UAL 185 and UAL 275 had patterns that are were 82% homologous with *Lactobacillus sakei* DUP-5032 and this represented the closest species match in their database. The UAL 185 and 275 patterns compared with one another had 87% homology and aside from one additional band pattern for UAL 275 in Figure 6A the RiboPrint patterns were identical. The *L. lactis* control strain ATCC 11454 had 81% homology with the database pattern for *Lactococcus lactis* (Figure 6B).

Table 4 Carbohydrate fermentation pattern (after 48 hours) of selected strains of lactic acid bacteria according to API CH50 (data obtained from the second determination).

Carbohydrate Test	UAL 185	ATCC 11454	UAL 187	UAL 280
Control	-	-	-	-
Glycerol	-	-	-	-
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	+	-	+	+
Ribose	+	+	+	+
D-Xylose	-	-	+	+
L-Xylose	-	-	-	-
Adonitol	-	-	-	-
β-Methyl-D-Xyloside	-	-	-	-
Galactose	+	+	-	+
Glucose	+	+	+	+
Fructose	+	+	+	+
Mannose	+	+	+	+
Sorbose	-	-	-	-
Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	-	-	-	-
Sorbitol	-	-	-	-
α-Methyl-D-Mannoside	-	-	-	-
α-Methyl-D-Glucoside	-	-	-	+
N-Acetyl-Glucosamine	+	+	+	+
Amygdalin	-	-	-	-
Arbutin	-	-	-	-
Esculin	+	+	+	+
Salicin	+	+	-	-
Cellobiose	+	+	+	+
Maltose	+	+	+	+
Lactose	+	-	-	-
Melibiose	+	-	+	+
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Inulin	-	-	-	-
Melezitose	-	-	-	-
Raffinose	-	-	+	-
Starch	-	+	-	-
Glycogen	-	-	-	-
Xylitol	-	-	-	-
Gentiobiose	+	-	+	+
D-Turanose	-	-	+	+
D-Lyxose	-	-	-	-
D-Tagatose	-	-	-	-
D-Fucose	-	-	-	-
L-Fucose	-	-	-	-
D-Arabitol	-	-	-	-
L-Arabitol	-	-	-	-
Gluconate	+	-	-	+
2-Keto-Gluconate	-	-	-	-
5-Keto-Gluconate	-	-	-	+

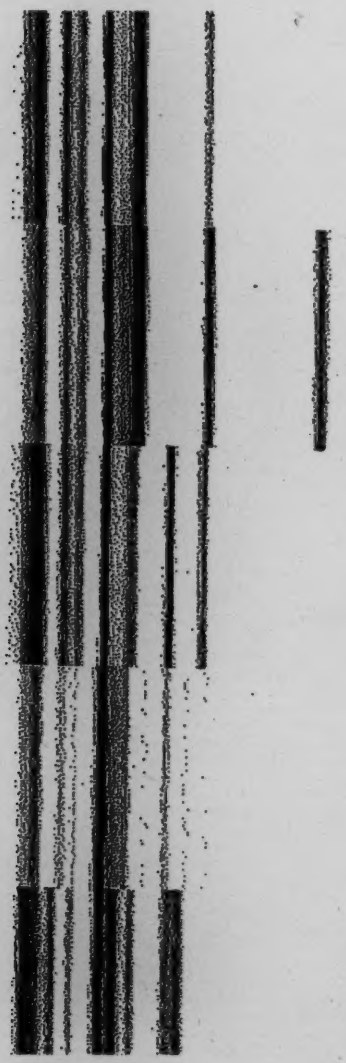
Strain	Riboprint Pattern
Strain UAL 185	
Strain UAL 275	
Lactobacillus sakei DUP-5032	
Lactobacillus sakei DUP-5033	
Lactobacillus curvatus DUP-5019	

Figure 6A Riboprint patterns of UAL 185 and UAL 275, compared with homologous patterns in the RiboPrinter® Microbial Characterization System.

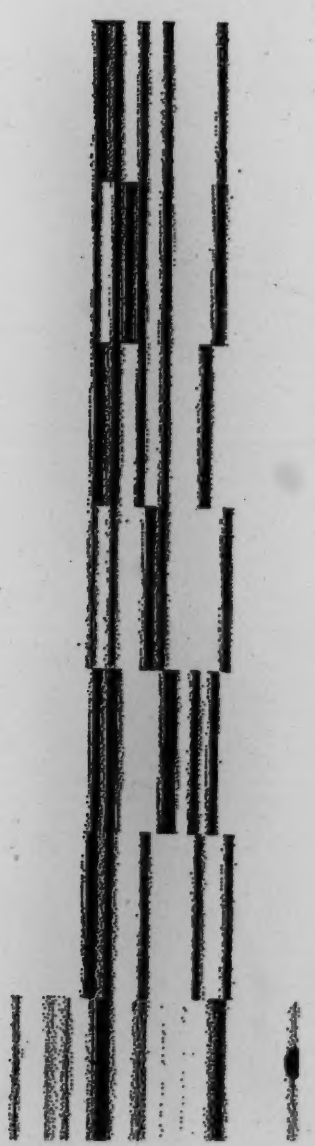
Strain	Riboprint Pattern
L. lactis ATCC 11454	
L. lactis DUP-5208	
L. lactis DUP-5204	
L. lactis DUP-5210	
L. lactis DUP-5206	
L. lactis DUP-5209	
Brevibacillus laterosporus DUP-11127	

Figure 6B Riboprint pattern of *L. lactis* ATCC 11454, compared with homologous patterns in the RiboPrinter® Microbial Characterization System.

This homology is comparable to the level of homology observed for the UAL 185 and 275 strains with *L. sakei*. Based on these data it was decided to confirm the identity of strain UAL 185 by sequencing the 16S rDNA.

4.3.3 16S rDNA Sequencing

Chromosomal DNA was isolated and the concentration and purity was examined by running 1 μL samples on a 1% agarose gel. DNA was subsequently diluted 1:10, 1:50, 1:100, and 1:200 and added to PCR mixtures to amplify the 789-bp fragment with PC3mod and POmod primers and the 721-bp fragment with PC5 and P3mod primers, as described in Materials and Methods (Section 3.3.3). Amplification data are shown in Figure 7. A 100-bp marker confirmed amplification of the desired size of the 16S rDNA fragment. The 721-bp fragment amplified with DNA diluted 1:100 and 1:200 did not amplify successfully. The purified PCR products from DNA in lanes 3 and 7 were sent for sequencing at the University of Alberta Medical Sciences Sequencing Laboratory. Sequencing data were analyzed for inconsistencies and the sequences were submitted to GenBank for a BLAST homology search of known nucleotide sequences in the database. The results indicated that the UAL 185 16S rDNA sequence was most homologous with the database sequences for DNA for 16S rRNA of *Lactobacillus sakei* and *Lactobacillus casei*. The sequence homology to *L. sakei* subsp. *sakei* DSM 20017 was 99% and the sequence homology to *L. casei* subsp. *fusiformis* strain DSM 20013 was 99%. The DSM 20013 strain has been recently reclassified as *L. paracasei* subsp. *paracasei* DSM 20006 and GenBank has not been updated with the new classification. The next closest homology (96%) was to a *C. piscicola* strain.

4.4 Plasmid Studies

4.4.1 Plasmid Curing and Variant Screening

Based on the report by Mishra (1992), it was expected that the strains would contain plasmid DNA. Many bacteriocins produced by LAB are plasmid-encoded. Consequently, it was decided to subject the strain to the novobiocin plasmid curing

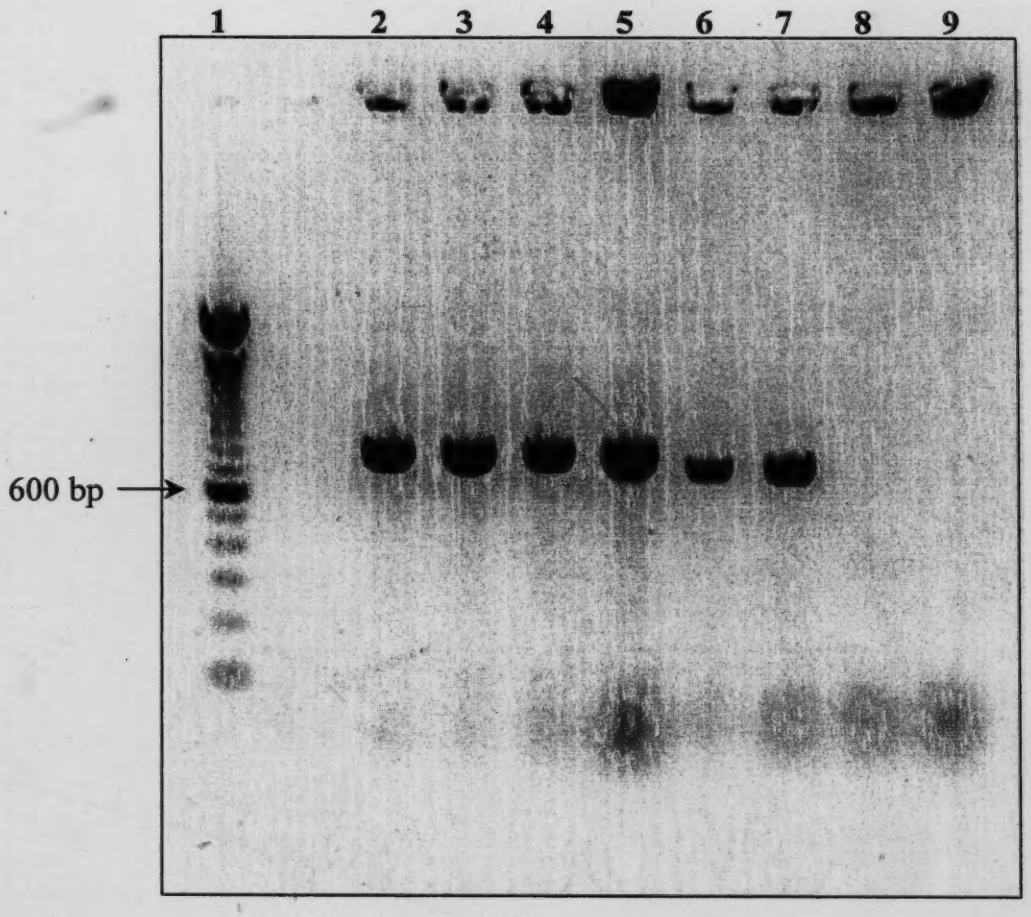


Figure 7 Fragments of UAL 185 16S rDNA amplified by PCR and analyzed on 1.8% agarose gel.
Lanes 2 to 5: amplification products of the 789-bp fragment of 16S rDNA, dilutions of chromosomal DNA; 1:10, 1:50, 1:100 and 1:200.
Lanes 6 to 9: amplification products of the 721-bp fragment of 16S rDNA, dilutions of chromosomal DNA; 1:10, 1:50, 1:100 and 1:200.

technique as described in Materials and Methods (See Section 3.4.1). Colonies were screened by deferred inhibition assay for loss of antimicrobial activity, throughout the incubation period. Antibacterial activity was detected in all of the colonies of UAL 185 that were tested.

4.4.2 Small-scale Plasmid Isolation

At the same time as the plasmid curing experiments, mini-prep plasmid isolations were done to check for the presence of plasmid DNA. Using the modified Birnboim and Doly protocol (1979) described in Materials and Methods, plasmid DNA was detected for the UAL 185 strain (Figure 8). The size of the plasmid DNA band was approximately 10 MDa, compared to the reference strains used in the study. The band was not clearly visible, compared to the reference strains and this could be due to a low copy number for this plasmid. The plasmid profile for the reference strain *L. gelidum* UAL 187 showed three plasmid DNA bands, corresponding to the 5.0, 7.6, and 9.2 MDa sizes reported in the literature (Hastings and Stiles, 1991). However, van Belkum and Stiles (1995) determined that the two largest plasmids of *L. gelidum* UAL 187 were 18 and 21 kb, respectively, which corresponds to approximately 12 and 15 MDa, respectively. The plasmid profile for *C. piscicola* UAL 8 showed one plasmid DNA band that corresponds with the 9.5 MDa plasmid reported in the literature (Ahn and Stiles, 1990). Ahn and Stiles (1990) reported four plasmids in the *C. piscicola* UAL 8 strain that were 9.5, 23.5, 40, and 49 MDa in size. The profile in Figure 8 shows only the small plasmid because the protocol used is not suitable for large plasmids. This might also explain the lack of largest plasmids of *L. lactis* ATCC 11454 in Figure 6. The reference strain *L. lactis* ATCC 11454 showed 3 plasmid bands (Figure 6) corresponding to the 1.4, 3.6, and 19.4 MDa plasmids reported in the literature (Gonzalez and Kunka, 1985). The 19.4 MDa band might also consist of the 21.9 MDa plasmid reported by Gonzalez and Kunka (1985). As expected, the plasmidless strain *C. piscicola* UAL 26 did not show any plasmid DNA on the gel.

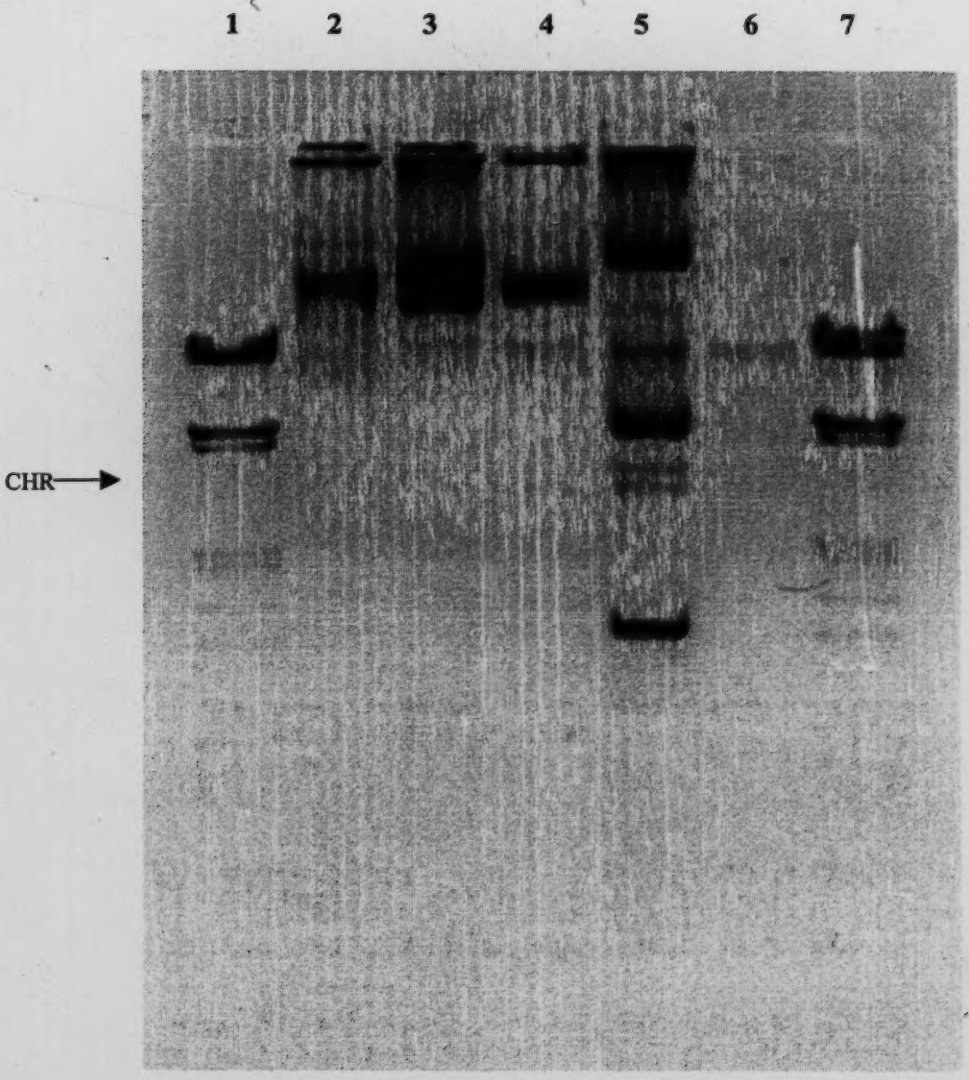


Figure 8 Plasmid profile of lactic acid bacteria strains on 1% agarose gel
Lane 1 and 7 = Lambda DNA cut with *Eco*RI and *Hind* III;
Lane 2 = UAL 185; Lane 3 = *L. gelidum* UAL 187;
Lane 4 = *C. piscicola* UAL 8; Lane 5 = *L. lactis* ATCC 11454;
Lane 6 = *C. piscicola* UAL 26.
CHR = Bands of chromosomal DNA

4.4.3 Large-scale Plasmid Isolation

The plasmid DNA seen in the small-scale plasmid isolation was difficult to assess based on the assumption that it is probably present in low copy number. Therefore, large-scale plasmid isolation procedures were attempted to isolate greater amounts of plasmid DNA. After the ultracentrifugation, a large fuzzy band of DNA was observed in the cesium-chloride gradient. It was not possible to separate the chromosomal and plasmid DNA bands in the gradient. Therefore, the entire band was extracted and dialyzed prior to precipitation of the DNA. A 1.5% agarose gel was run and a band of chromosomal DNA was observed. No plasmid bands were observed on the gel.

4.5 Concentration of Antimicrobial Substance(s)

The antimicrobial substance(s) produced by UAL 185 could not be detected in the supernatant of a fully grown culture by spot-on-lawn inhibition technique. Therefore, in order to characterize the inhibitory substance(s) it was necessary to concentrate it from the culture supernatant. Two techniques for preliminary concentration of inhibitory substance(s) were in use in the laboratory at the time: 1) Hydrophobic exchange column technique; and 2) Butanol extraction

4.5.1 XAD-8 Hydrophobic Exchange Column

The supernatant fraction from a UAL 185 fermentation was heat treated and applied to an XAD-8 hydrophobic interaction column. A sample taken before concentration showed no antimicrobial activity. The column was eluted with 40%, 70%, and 95% ethanol and these fractions were concentrated by rotary evaporation and tested for antimicrobial activity. There was no activity detected in any of the fractions when assayed against *C. divergens* UAL 9 as the indicator organism. *L. gelidum* UAL 187 was used as a reference. Spot-on-lawn assays detected leucocin activity of 12,800 AU(arbitrary activity units)/mL from the 70% ethanol fraction and 3,200 AU/mL from the unconcentrated supernatant sample.

4.5.2 Butanol Extraction

The three butanol extractions (total 1.5 L) were pooled and concentrated by rotary evaporation. Butanol extractions of the 2L supernatant from UAL 185 yielded an activity of 200 AU/mL when measured against *C. divergens* UAL 9 as the indicator organism. The spotted sample from the butanol extraction was coincubated with a 10 mg/mL solution of Pronase E and the activity was not inactivated. This indicated that the active substance(s) was not proteinaceous. A control spot of butanol was spotted onto the indicator plate and did not cause inhibition of the indicator organism. Production of brochocin-C by *B. campestris* ATCC 43754 was used as a reference. The butanol extract of the supernatant from this strain resulted in the recovery of 819,200 AU/mL when measured against *C. divergens* UAL 9 as the indicator organism. The activity of *B. campestris* 43754 in the supernatant prior to the butanol extraction was 12,800 AU/mL.

5. DISCUSSION

Biopreservation refers to extended storage life and enhanced safety of foods using the natural microflora and (or) their antibacterial products (Stiles, 1996). LAB are promising candidates for biopreservation because they have been used for long periods of time to produce various fermented foods from products derived from animals or plants. There are instances of food spoilage due to LAB, but their essential and desired role in preservation and flavour production in fermented foods is of far greater interest than the spoilage potential.

In recent years, much research has focused on the use of LAB to preserve foods and to inhibit foodborne pathogens. This study evaluated the efficacy of a strain of LAB against the foodborne pathogen *L. monocytogenes* on a processed meat product. *L. monocytogenes* is commonly found on many different food products. A report by the World Health Organization quoted that '*Listeria monocytogenes* is a widely distributed environmental contaminant whose primary means of transmission to humans is through contamination of foodstuffs at any point in the food chain, from source to kitchen (Bell and Kyriakides, 1998). The total elimination of *Listeria monocytogenes* from all food is impractical and may be impossible'. The natural exposure of vegetables, fruits, grains, food animals, fish and poultry to the environmental sources of the organism makes it impossible for foods to be produced free from *L. monocytogenes* at source. For example, *Listeria* spp. were detected on 93 of 175 samples of vacuum-packaged processed meats obtained from 15 retail stores over a 12-month period in Australia. It was also found in the study that 5% of the samples contained more than 1,000 cfu per gram (Grau and Vanderlinde, 1992). Johnson et al. (1990) reported prevalence ranges in of *L. monocytogenes* from 5 to 13% in ready-to-eat meat products, in which typical plate counts ranged from less than 10 to 1,000 CFU per gram. Other studies have reported varying occurrence rates but overall it is apparent that the presence of listeriae in meat products is a problem in the food industry and must be addressed at specific stages in meat processing.

The different characteristics that make *L. monocytogenes* an organism of importance for the food industry and public health are its psychrotrophic growth characteristics, high mortality rate associated with documented cases of foodborne listeriosis and increasing evidence that sources of contamination of food are from the environment (Farber and Peterkin, 1991). Therefore, there has been increasing interest in targeting *L. monocytogenes* in food biopreservation. Methods utilized by industry to reduce bacterial loads, such as spraying carcasses with antimicrobials or food irradiation are not targeted specifically to pathogens such as *L. monocytogenes* and therefore the elimination of all microbes may create an environment that is more susceptible to the growth of pathogens. Biopreservation techniques may be effective in targeting specific pathogens but their efficacy must be assessed in a specific food product because demonstration of effectiveness in one food system may not necessarily be true for another.

The experimental strains, UAL 185 and 275 used in this study were isolated by Mishra (1992) from vacuum-packaged, chill-stored fresh beef. Isolates from the meat products were screened for production of antagonistic compounds and it was reported that these strains exhibited promising characteristics due to their ability to grow at low temperatures in a meat environment, and inhibit a broad spectrum of pathogens, similar to the spectrum of antimicrobial activity reported for nisin. In this study, the broad antibacterial spectrum of UAL 185 was confirmed and it was shown to be comparable to the activity of the nisin-producing strain of *L. lactis* ATCC 11454. Nisin is used as a common reference to evaluate bacterial antimicrobials due to its broad activity spectrum and its acceptability for use in the food industry. Nisin has been shown to be ineffective as an antibacterial agent in raw meats, so the original interest in UAL 185 was due to its antibacterial activity and psychrotrophic nature. UAL 185 inhibited all pathogens and non-pathogens used as indicators in this study when grown on Lactobacilli MRS agar at 25°C under both aerobic and anaerobic conditions. These results indicate that UAL 185 may function in both anaerobic (vacuum-packaged) and aerobic conditions used in meat packaging.

The results of physiological and biochemical characterization in the previous study supported the conclusion that UAL 185 and probably UAL 275 be placed in the genus

Lactococcus. This was a surprising conclusion considering that *Lactococcus* spp. are not generally isolated from meat products and strains of *L. lactis* that grow at refrigeration temperatures have not been reported. Therefore the present study investigated these claims and evaluated the efficacy of UAL 185 and UAL 275 on a processed meat product for its ability to inhibit three strains of *L. monocytogenes*. Initially, the API 50CH carbohydrate fermentation profile of UAL 185 confirmed that this organism was *Lactococcus lactis*. It was subsequently observed that the cellular morphology did not agree with the results previously reported. In the present study it was observed that UAL 185 was a blunt rod-shaped, Gram-positive organism unlike a characteristic coccus-shaped lactococcus. However, by this time the challenge study against *L. monocytogenes* had been completed and the antibacterial spectra of UAL 185 and UAL 275 had been confirmed. The UAL 275 organism was shown to be a polymorphic colony variant of UAL 185. Therefore, the data for UAL 275 in the challenge study represent a duplicate of the results obtained for UAL 185.

The challenge study demonstrated the ability of UAL 185 and UAL 275 to inhibit the growth of the *L. monocytogenes* cocktail inoculated on the surface of a processed wiener product that was vacuum packaged and stored at 4°C for 14 weeks. Wieners were chosen as a model because of the potential for postprocessing contamination between peeling and packaging of the product (Berry et al., 1991). It is known that there is an epidemiological association of listeriosis and ingestion of non-reheated wieners (Schwartz et al., 1988), therefore the wiener product used in the study is a meat product where *L. monocytogenes* is a pathogen of concern. The experimental procedure for this study represented a practical post-processing surface contamination that could occur in industry, although for experimental purposes inoculum levels with *L. monocytogenes* were considerably higher than would realistically occur during production. Reports indicate that typical counts of *L. monocytogenes* range from <10 to 1,000 cfu/g in raw and processed meat products (Buchanan et al., 1989; Johnson et al., 1990). The surface inoculation procedure achieved the desired levels of *L. monocytogenes* and the LAB cultures on the product. In both replicates the background microflora of the wieners was 100-fold lower than the LAB inoculum level. In the two replicates of the challenge

study, the cocktail of *L. monocytogenes* strains grew to a maximum population of 7 log cfu/cm^2 within 14 weeks of storage at 4°C, and the background microflora on the uninoculated control samples also grew to 7 log cfu/cm^2 within 14 weeks. In contrast, when the inoculated LAB strains grew on the wieners, the *L. monocytogenes* inoculum did not grow and remained at or slightly below 3 log cfu/cm^2. This indicates that the LAB were sufficient to outnumber the natural microflora of the product and to inhibit the growth of the inoculated strains of *L. monocytogenes*. In the co-inoculation treatments with *L. monocytogenes* and UAL 185 or UAL 275 the inhibitory effect was observed throughout the 14-week storage period.

The experimental wieners were produced by a commercial manufacturer with a one-week interval between replicates, so there was no "absolute" control over the characteristics of the product. This may account for the difference in pH profiles of the product during storage and could cause a difference in the composition of the background microflora on the wieners. All other experimental characteristics in the laboratory were identical between replicates. The fact that the *L. monocytogenes* cocktail was inhibited on the wieners in both replicates, despite inherent differences in the product, is considered a strength rather than a weakness of the study. The difference between replicates emphasizes the need for additional replicates in future challenge studies.

The bioprotective effect of these strains was apparent from the greater than 4 log difference in numbers of *L. monocytogenes* between the samples with *L. monocytogenes* alone and those with added LAB. The LAB cultures inhibited growth of *L. monocytogenes* on the wieners but did not decrease bacterial numbers and therefore they had a bacteriostatic rather than the desired bactericidal effect. The inhibitory effect could not be directly related to the inhibitory substance(s) produced by UAL 185 and UAL 275. Curing experiments to obtain an isogenic strain of UAL 185 were not successful. It was subsequently shown that UAL 185 probably does contain plasmid DNA by a small-scale plasmid isolation procedure, however the one visible band contradicts the information reported by Mishra (1992). Although no obvious undesirable sensory characteristics

were observed with the treatments, further sensory investigations are required to assess changes in product quality induced by UAL 185.

It was reported by Schillinger et al. (1991) that a strain of *L. sakei* inoculated in ground pork (German-type fresh Mettwurst) suppressed the growth of *L. monocytogenes*. It was also reported that the bacteriocin-producing *L. sakei* Lb706 reduced the count and delayed the growth of listeria more effectively than the bacteriocin-negative strain. A strain of *L. sakei* inoculated onto chicken cold cuts, vacuum packaged and stored at 4 and 10°C for 4 weeks showed an inhibitory effect against *L. monocytogenes* for both the bacteriocin producer and the negative isogenic variant (Katla et al., 2002). Therefore, the authors concluded that the inhibitory effects of *L. sakei*, other than bacteriocin production, are active in inhibiting the growth of *L. monocytogenes* on chicken cold cuts. A recent study by Amezquita and Brashears (2002), investigated the effect of three strains of LAB, co-inoculated with *L. monocytogenes* on frankfurters and cooked ham, vacuum packaged and stored at 5°C for 28 days. Bacteriocidal activity was observed in the frankfurter samples where a 4.2 to 4.7 log cfu/cm^2 decrease in *L. monocytogenes* counts, compared to the controls, was observed (Amezquita and Brashears, 2002). This is a comparable decrease to the *L. monocytogenes* inhibition found in the present challenge study, however in this study the LAB (*P. acidilactici*, *L. casei*, *L. paracasei*) counts increased by only one log cycle. The authors concluded that the inhibitory effect was due to bacteriocin production by the *P. acidilactici* strain and organic acid production by the *Lactobacillus* spp. strains. The ability of LAB to grow and inhibit pathogens in meat products is a combination of effects and therefore all inhibitory actions must be included when evaluating a strain as a potential biopreservative culture.

Leisner et al. (1995) examined the effect of LAB in extending the storage life of beef and concluded that the *Leuconostoc gelidum* UAL 187 strain showed good potential. All four LAB tested did not cause spoilage of the meat during the 10-week storage period under vacuum. In a later study, chill-stored, vacuum packaged beef was inoculated with a sulfide-producing spoilage strain of *L. sakei* 1218, and co-inoculated with bacteriocinogenic *L. gelidum* UAL 187. *L. gelidum* UAL 187 delayed the spoilage by *L.*

sakei 1218 for up to 8 weeks of storage (Leisner et al., 1996). However, *L. gelidum* UAL 187 produces dextran from sucrose which limits its application in processed and/or cured meats with added sucrose (McMullen and Stiles, 1996). This study demonstrated the ability of LAB to inhibit spoilage organisms. Food spoilage inhibition is also important to assess because it can be beneficial in the improvement of product quality and the extension of product shelf life. It would be beneficial to evaluate the effectiveness of UAL 185 in inhibiting meat spoilage organisms, such as *Brochothrix* spp. or other strains of *L. sakei* that have been reported to cause spoilage characteristics, such as slime formation and off-odours in meats.

The challenge study showed that UAL 185 was effective in inhibiting *L. monocytogenes* on wieners and therefore complete identification of the strain was necessary. The results of biochemical typing were not definitive. Morphological and physiological characteristics measured in this study were not consistent (rod shaped, heterofermentative and psychrotrophic) with *L. lactis*. Therefore it was decided to confirm the identity of UAL 185 by genetic typing methods such as ribotyping and 16S rDNA sequence determination.

Ribotyping provides a genetic "fingerprint" or RiboPrint pattern for bacterial isolates based on a patented technology that uses patterns derived from universal probes targeted at specific conserved domains of the ribosomal RNA by comparison with reference patterns in the RiboPrint Pattern Identification Database (www.sph.umich.edu/macepid/ resources/ribotyping .html). It is claimed that the results are unequivocal unlike the subjective results obtained from biochemical-based tests or other manual typing methods. In one study researchers established ribotypes of beer-spoilage strains of *Lactobacillus* spp. for strain discrimination purposes and concluded that ribotyping can be used for species discrimination from the set of conserved ribotyped fragments, and can also be used for strain typing from polymorphic variations of ribotyping (Motoyama et al., 2000). Based on ribotyping data, strain UAL 185 was identified as *L. sakei* because it had greatest homology (82%) with *L. sakei* strain DUP-5032 in the riboprint reference collection.

To confirm this identification, 16S rDNA sequencing was also done on strain UAL 185. The 16S rDNA sequence is highly conserved among bacteria. Mutations in the 16S rDNA genes have occurred at a slow but constant rate over time and these mutations are usually located in characteristic divergent or variable regions of the molecule (Woese, 1987). By sequencing the divergent regions in the gene and comparing these sequences to a database of known 16S rRNA sequences, identification of a bacterial isolate can be accurately achieved (Weisberg, et al., 1991). The 16S rDNA sequence for UAL 185 had greatest homology with *L. sakei* DSM 20017 and *L. casei* DSM 20013 in the GenBank database. It appears that the 16S rDNA sequencing results are not as definite as the ribotyping technique. Because UAL 185 was identified as having high homology with *L. sakei* by both methods it was concluded that this strain is most probably *L. sakei*.

The experimental strain *L. sakei* UAL 185 was originally isolated from "vacuum packaged and chill stored beef" (Mishra, 1992) and it was confirmed to be psychrotrophic. The UAL 185 growth rate at 1°C in broth was similar to the growth rate of four other psychotrophic bacteria (two *Carnobacterium* spp. and two *Leuconostoc* spp.) that were isolated from meat and tested under the same experimental conditions. The ability of UAL 185 to grow well at 1°C shows its potential for use as a bioprotective culture in food products stored at cold temperature. The organism also grew well at 25°C with a generation time of approximately 1 hour, making this a good temperature at which to grow the culture for large-scale production.

Antimicrobial activity could not be detected in unconcentrated culture supernatant, therefore attempts were made to concentrate the active component. The hydrophobic interaction exchange column failed to concentrate the inhibitory substance(s) produced by the test strains; however butanol extraction successfully concentrated a small amount of antimicrobial substance(s). However, the compound concentrated by butanol extraction was not proteinaceous so further study is required to identify the nature of the inhibitory substance(s).

The present study confirmed the activity spectrum of UAL 185, but it is not possible to speculate on the identity of the antimicrobial substance(s) that were detected in *in vitro* assays and in the challenge study. Additional challenge studies should be done to confirm the inhibitory effect against a broader spectrum of strains of *L. monocytogenes,* especially strains associated with foodborne infections. The *Listeria* spp. cocktail used in the current study was developed and characterized by Panayach (1998), but it does not include all serotypes of *L. monocytogenes* that have been associated with foodborne illness. It is also important to assess the inhibitory activity of UAL 185 in other meat products. Processed meats have differing formulations and therefore it cannot be assumed that effectiveness in one product will ensure a similar effect in another.

The effect of different inoculum levels of UAL 185 in meat products must also be investigated to maximize the inhibitory and preservative effect. In approving an organism for use in a food product it is also important to assess whether the addition of the culture to the food product results in any deleterious effects. Sensory studies to assess product quality must be done before marketing a food with a biopreservative agent. Food processors must have assurance that a product inoculated with a biopreservative culture will be of acceptable quality when introduced into the market because it is ultimately the consumer that decided whether a product will succeed in the marketplace.

In conclusion, UAL 185 was shown to be a good strain of *L. sakei* to inhibit *L. monocytogenes* on vacuum packaged wieners; however, more information about the inhibitory action and the effect of UAL 185 on organoleptic properties of the wieners must be studied before the use of this organism as a biopreservative can be recommended.

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7. APPENDIX

Table 7.1 Least Square Means log cfu/cm^2 of presumptive lactic acid bacteria counts on APT from blended wiener samples, vacuum packaged and stored at 4°C for 14 weeks.

Table 7.2 Least Square Means log cfu/cm^2 of *L.* monocytogenes counts on PALCAM from blended wiener samples, vacuum packaged and stored at 4°C for 14 weeks.

Table 7.3 Least Square Means pH measurements for blended wiener samples, vacuum packaged and stored at 4°C for 14 weeks.

Table 7.1 Least Square Means[1] log cfu/cm^2 of presumptive lactic acid bacteria plate counts on APT from blended wiener samples, vacuum packaged and stored at 4°C for 14 weeks.

STORAGE TIME (WEEKS)	TREATMENTS							
	Control	185	275	187	185 + Listeria	275 + Listeria	Listeria Control	SEM[2]
0	2.29^{b}	4.21^{a}	3.78^{a}	3.91^{a}	3.70^{a}	3.86^{a}	2.73^{b}	0.19**
1	2.41^{b}	5.50^{a}	5.87^{a}	4.98^{ab}	4.08^{ab}	4.38^{ab}	3.92^{ab}	0.56*
2	2.55^{b}	7.34^{a}	7.03^{a}	5.58^{a}	6.99^{a}	7.14^{a}	5.37^{a}	0.39***
3	2.50^{d}	9.26^{a}	8.99^{ab}	5.94^{c}	8.53^{ab}	8.19^{b}	5.86^{c}	0.20***
4	3.35^{c}	8.78^{a}	8.22^{a}	6.58^{b}	8.31^{a}	7.96^{a}	5.96^{b}	0.22***
5	4.93^{b}	8.53^{a}	8.24^{a}	7.04^{a}	8.49^{a}	8.19^{a}	7.08^{a}	0.31***
6	5.94^{b}	8.41^{a}	8.10^{a}	7.31^{a}	8.37^{a}	8.15^{a}	7.36^{a}	0.27**
7	5.26^{c}	8.39^{a}	8.13^{a}	7.05^{b}	8.31^{a}	7.95^{a}	7.63^{ab}	0.21***
8	5.18^{b}	8.36^{a}	8.05^{a}	7.17^{a}	8.26^{a}	7.99^{a}	7.97^{a}	0.23***
10	6.54^{b}	8.44^{ab}	8.03^{ab}	7.74^{ab}	8.76^{a}	8.47^{ab}	7.89^{ab}	0.37*
12	6.49^{b}	8.35^{a}	7.88^{a}	8.02^{a}	8.16^{a}	8.37^{a}	7.91^{a}	0.21**
14	7.10	8.25	7.76	7.37	8.06	7.81	8.00	0.35$_{NS}$

1 Least square means were calculated from the average count for each replicate that was determined from duplicate counts for each sample

2 Standard error of the mean

abcd Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

NS Non-significant difference between treatments

* Significant at $p<0.05$

** Significant at $p<0.01$

*** Significant at $p<0.001$

Table 7.2 Least Square Means[1] log cfu/cm^2 of *Listeria* spp. plate counts on PALCAM from blended wiener samples, vacuum packaged and stored at 4°C for 14 weeks.

STORAGE TIME (WEEKS)	TREATMENTS							
	Control	185	275	187	185 + *Listeria*	275 + *Listeria*	*Listeria* Control	SEM[2]
0	<1.00^b	<1.00^b	<1.00^b	<1.00^b	2.51^a	2.62^a	2.79^a	0.12•••
1	<1.00^c	<1.00^c	<1.00^c	<1.00^c	2.74^b	2.62^b	3.73^a	0.24•••
2	<1.00^d	<1.00^d	<1.00^d	<1.00^d	3.20^c	3.53^b	4.96^a	0.07•••
3	<1.00^d	<1.00^d	<1.00^d	<1.00^d	3.60^b	2.97^c	5.07^a	0.17•••
4	<1.00^c	<1.00^c	<1.00^c	<1.00^c	3.15^b	2.94^b	5.05^a	0.34•••
5	<1.00^c	<1.00^c	<1.00^c	<1.00^c	3.13^b	3.26^b	6.12^a	0.51•••
6	<1.00^b	<1.00^b	<1.00^b	<1.00^b	2.62^b	2.76^b	6.56^a	0.57•••
7	<1.00^c	<1.00^c	<1.00^c	<1.00^c	3.51^b	2.93^b	7.38^a	0.53•••
8	<1.00^b	<1.00^b	<1.00^b	<1.00^b	2.73^b	2.63^b	7.68^a	0.52•••
10	<1.00^c	<1.00^c	<1.00^c	<1.00^c	2.54^b	2.68^b	7.61^a	0.34•••
12	<1.00^c	<1.00^c	<1.00^c	<1.00^c	2.58^b	2.62^b	7.61^a	0.40•••
14	<1.00^c	<1.00^c	<1.00^c	<1.00^c	2.18^b	1.86^b	7.04^a	0.22•••

1 Least Square Means were calculated from the average count for each replicate that was determined from duplicate counts for each sample

2 Standard error of the mean

abcd Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

NS Non-significant difference between treatments

* Significant at $p<0.05$

** Significant at $p<0.01$

*** Significant at $p<0.001$

Table 7.3 Least Square Means[1] pH measurements for blended wiener samples, vacuum packaged and stored at 4°C for 14 weeks.

STORAGE TIME (WEEKS)	TREATMENTS							
	Control	185	275	187	185 + Listeria	275 + Listeria	Listeria Control	SEM[2]
0	6.42	6.37	6.40	6.43	6.44	6.42	6.44	0.04_{NS}
1	6.44	6.47	6.46	6.51	6.47	6.45	6.49	0.03_{NS}
2	6.42	6.42	6.40	6.49	6.45	6.44	6.47	0.02_{NS}
3	6.40	6.19	6.24	6.49	6.29	6.27	6.46	0.09_{NS}
4	6.45^{a}	5.82^{a}	6.03^{a}	6.43^{a}	6.06^{a}	6.13^{a}	6.47^{a}	0.13*
5	6.52	5.72	6.06	6.39	5.85	5.98	6.48	0.16_{NS}
6	6.37^{a}	5.48^{b}	5.83^{ab}	6.35^{a}	5.62^{ab}	5.87^{ab}	6.42^{a}	0.17*
7	6.41	5.38	5.68	6.27	5.57	5.73	6.40	0.26_{NS}
8	6.23	5.24	5.56	6.11	5.28	5.56	6.03	0.23_{NS}
10	6.23	5.02	5.22	5.87	4.93	5.41	5.94	0.27_{NS}
12	6.11	4.91	5.17	5.39	5.03	5.26	5.62	0.36_{NS}
14	5.75	4.85	5.14	5.33	4.96	5.30	5.48	0.37_{NS}

1 Least Square Means were calculated from the average count for each replicate that was determined from duplicate counts for each sample

2 Standard error of the mean

abcd Means within the same row sharing a common letter are not significantly different from each other, $p<0.05$

NS Non-significant difference between treatments

* Significant at $p<0.05$

** Significant at $p<0.01$

*** Significant at $p<0.001$